Application of supercritical fluid chromatography and extraction in pharmaceutical and environmental analysis

Fischer, Monika

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Application of Supercritical Fluid Chromatography and Extraction in Pharmaceutical and Environmental Analysis

Submitted by Monika Fischer
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Summary

The application of SFC for the separation of tobacco alkaloids of different basicity strength has been demonstrated on various columns, namely (S)-NEC-β-CD, β-CD, diol and silica column. Basic additives were required for an efficient separation on all columns, precluding the use of a NPD detector. The investigation demonstrated the flexibility and ease of SFC to influence chromatographic parameters. However, the retention of anabasine and nornicotine was susceptible to small changes in the physical parameters and the presence of water. The variation was mainly due to the lack of a suitable amine additive which would have been sufficient in controlling the retention mechanism of anabasine and nornicotine.

A novel application of a (S)-NEC-β-CD column in separating PCBs according to their planarity was demonstrated, which was successful for all of the PCBs studied. When a more complex Aroclor 1260 mixture was fractionated, it was revealed that a number of PCBs had in fact eluted in the planar fractionation, despite being non-planar. The fractionation of the PCB mixture also highlighted the problem of PCBs precipitation in the backpressure needle valve, therefore the set-up of the SFC needs to be further optimised for quantitative off-line collection.

A unique method for relating toxicity to the retention on a (S)-NEC-β-CD column was proposed by calculating retention ratios for the PCBs on the (S)-NEC-β-CD column relative to β-CD, diol and silica column, which expresses the retention enhancement due to π-π interactions. The ratio between the (S)-NEC-β-CD and silica column correlated best with toxicity, however the model could not be extended to other chemicals, as toxicity was not purely dependent on the interaction strength of compounds with the receptor. Nevertheless, the ratios of the compounds used in this work indicated that compounds with endocrine

disrupting properties might be identified using this method. When further validation is conducted, this would allow a very fast and efficient technique for screening numerous chemicals.

Furthermore, SFC can be used successfully for the separation of PCBs from fat. Four columns were investigated, of which silica was the most efficient, achieving the separation within 5 minutes. The silica column was also the most efficient in achieving the separation of PAHs from fat and sunflower oil within minutes.

Additionally, it was shown that SFC can be applied in the investigation of suppository mass. This highlighted the great potential of SFC for analysing both the suppository mass and added drug concomitantly.

Chiral separation of propanolol and clofibrate analogues was conducted using the chiral (S)-NEC-β-CD and β-CD column. SFC proved to be a very efficient technique and chiral separation of some clofibrate analogues was achieved in under 4 minutes, demonstrating the superiority of SFC over LC.

SFE of fatty acids from cotton seed meal and soya flour meal and of nicotine from tobacco was demonstrated. For fatty acid extraction a statistical design optimisation was used, which pinpointed areas of optimum conditions. An attempt to optimise the extraction of nicotine by varying individual physical parameters was carried out, however, it was found that the water content present in the extraction cell was a major factor in determining extraction recovery. The latter extraction highlighted the need for identifying all parameters which could influence the extraction and understanding their importance on extraction recovery.

List of Abbreviations

ASE	Accelerated Solvent Extraction
BTA	Butylamine
CBPs	Chemically Bonded Phases
CD	Cyclodextrin
CO_2	Carbon dioxide
CP	Critical Point
CP_M	Critical Point of Mixture
DDT	1,1-Di(4-chlorophenyl)-2,2,2-trichloro-ethane
DEA	Diethylamine
ECD	Electron capture detector
EOS	Equation of State
EPA	Environmental Protection Agency
FID	Flame Ionisation Detector
GC	Gas Chromatography
HPLC	High Performance (Pressure) Chromatography
HXA	Hexylamine
iso-PA	iso-Propylamine
LSD	Light Scattering Detector
MeCN	Acetonitrile
MeOH	Methanol
MS	Mass spectrometry

NP	Normal Phase
NPA	n-Propylamine
NPD	Nitrogen-Phosphorous Detector
OCA	Octylamine
ODS	Octyldecylsiloxane
PAHs	Polyaromatic Hydrocarbons
PCBs	Polychlorinated Biphenyls
2-PrOH	2-Propanol
PS-DVB	Polystyrene divinylbenzene
PYE	2-(1-pyrenyl)ethyldimethyl-silylated
RP	Reverse Phase
RSD	Relative Standard Deviation
SCF	Supercritical Fluid
SFC	Supercritical Fluid Chromatography
SFE	Supercritical Fluid Extraction
(S)-(NEC)	(S)-Naphthylethylcarbamate
TCDD	2,3,7,8-Tetrachloro-dibenzodioxin
TEA	Triethylamine
TEF	Toxic Equivalent Factor
TID	Thermionic Ionisation Detector
UV	Ultraviolet
V	critical Volume
ZPG	N-benzoxycarbonyl-glycyl-proline

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Finally, I wish to thank all the people who made the time in Bath an unforgettable experience. I wish to express special thanks to Micheal Matchett who was a great support during my first year and whose sense of humour alleviated a lot of frustration. I also want give special to thanks Dave Cross for sharing the laboratory for three years, which was a great experience. Last, but not least I would like to thank my future parents in law for their generous support during the writing of the thesis.

Widmung / Dedication

Ich möchte diese Arbeit meinem Vater und Mutter widmen für Ihre Unterstützung und daß ich mich immer auf Sie verlassen konnte.

Additionally, I would like to dedicate this work to Calum my future husband for his endless patience and support during the course of this work. He never doubted and was always encouraging.

Moni.

CHAPTER 1

INTRODUCTION

1.1 Historical Background of Supercritical Fluids (SCF)

Baron Cagnaird de la Tour was the first to discover the phenomenon of a critical point in 1822. He observed the discontinuity in sound when heating ether or carbon disulfide in a sealed cannon barrel while rocking the cannon barrel containing a flint ball and listening for the changes in the sound of the rolling ball. A change in sound was observed at the critical point. This was therefore referred to as the Cagnaird de la Tour point for many years.

Dr. Andrews conducted the first systematic study of the phase behaviour of supercritical fluid in 1869 and described the observation of the disappearance of the meniscus between liquid and vapour phase (Andrews 1876). An homogeneous fluid appeared at 30.92°C and 74.0bar which are in close agreement to the presently accepted values of 31.1°C and 73.8bar.

An initial account of the ability to dissolve solid material in supercritical fluid was given by Hannay and Hogarth at a meeting of the Royal Society of London in 1880 (Hannay and Hogarth 1880). They observed in their experiments that several inorganic salts such as cobalt chloride, potassium iodide and potassium bromide were soluble in ethanol above the critical temperature of ethanol.

This first report of the pressure dependence of supercritical fluid solvent power, however, was not without controversy. Prof. Ramsey reproduced the

experiments of Hannay and Hogarth and concluded that the observed phenomenon was merely the solubility of a solid in a hot liquid (Ramsey 1880).

Gore (1961) published the earliest work using liquid CO_2 as solvent for naphthalene and camphor. He found that the two compounds showed a limited solubility and concluded that liquid CO_2 is "a very feeble solvent of substances in general".

About the same time as the first solubilities of organic compounds in CO_2 were being investigated, Klesper et al. (1962) used dense gases as mobile phase for chromatography. They separated porphyrins and called this separation process "high pressure gas chromatography".

During the late 1970s and early 1980s there was much publicity in the USA concerning supercritical fluids, and SCF technology was regarded as a panacea for the chemicals, petroleum and food industries (McHugh and Krukonis 1994). Although numerous plants were commissioned in Europe for the extraction of coffee, tea, hops, spices and flavourings, the development of large scale processes in the USA lagged behind.

"Because of all the ill-advised and ill-fated developments of the early 1980s, and because of the sometimes desperate attempts of various research groups to garner publicity on the application of supercritical fluids to develop low cholesterol milk, meat, and liquid eggs, supercritical fluids are still today frequently met with scepticism whenever they are suggested as a solution to a real problem ...." (McHugh and Krukonis 1994)

As McHugh and Krukonis pointed out, the initial high hopes for supercritical fluids have not materialised, however, this is no reason to dismiss their potential for future applications.

Supercritical fluids are widely used throughout industry in such diverse applications as decaffeinating coffee, wastewater treatment and chemical

analyses. The potential use of supercritical CO_2 for polymer manufacturing, pharmaceuticals and soil remediation is currently being investigated.

At the University of North Carolina-Chapel Hill, CO_2 is used for producing fluoropolymers without the need for solvents and a technique has been developed to use it in producing micrometre-sized acrylic polymers (DeSimone et al. 1994). One of Europe's largest SFC-research groups, situated in Leeds, is investigating new methods of drug formulation, the preparation of sub-micron-sized particles and a new method of coating fine particles with polymers for controlled release (Riley 1994).

It is not possible to cite all the work currently taking place in supercritical fluid technology, however there are excellent introductory reviews available (Paulaitis et al. 1983 and Squire et al. 1987) and an outstanding survey book (McHugh and Krukonis 1994).

1.2 Fundamental Properties of Supercritical Fluids

In order to fully utilise the applications of supercritical fluids, it is vital to understand the basic properties and behaviour of these fluids. From initial simple investigations about phase behaviour, equations of states were derived and even today most of the equations of states rely on empirical constants.

1.2.1 Definition of Supercritical Fluids

The supercritical state of a substance is reached when both the critical temperature and pressure are exceeded and a distinction between liquid and gas phase can no longer be observed.

The phase diagram in Figure 1.1 shows the phase behaviour of a single substance characterised by its pressure curve, melting and sublimation curve

and the regions at which the phases are thermodynamically stable. The boundaries between the regions; the phase boundaries show the value of P and T at which two phases coexist in equilibrium. By following the vapour phase boundary, which extends from the triple point, TP, it is apparent that the vapour pressure increases with increasing temperature.

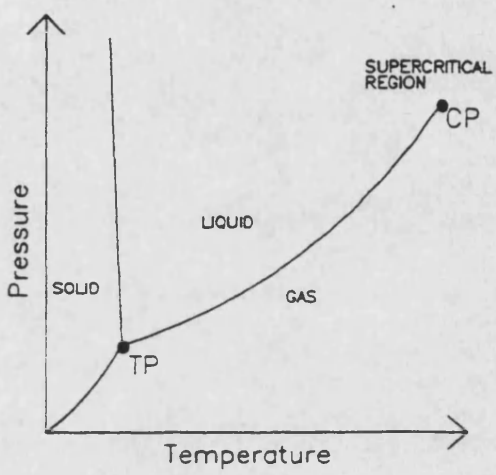


Figure 1.1 Phase diagram of a pure substance.

In practice this is achieved by heating a substance in a closed container. This causes a pressure increase, resulting in the gas phase having a higher density and being able to dissolve more liquid. Further continuous increase of temperature causes the liquid phase's density to decrease due to thermal expansion and the gas phase's density to increase, resulting in the two phases becoming increasingly similar. At and above the critical point, CP, the two phases merge into a single homogeneous phase. At a temperature higher than the critical temperature, T_c, and a pressure greater than the critical pressure, P_c, the homogeneous phase is said to be in the "super-critical region". In this state it is a fluid with changing properties from gas to liquid-like with increasing pressure and decreasing temperature.

Table 1.1 lists the critical temperatures and pressures of various solvents. As the critical temperatures of carbon dioxide, ethane and ethylene are near ambient temperature they have found wide acceptance as solvents for

processing heat-sensitive flavours, pharmaceuticals, labile lipids and reactive monomers (McHugh and Krukonis 1994).

Table 1.1 Critical parameters for selected fluids[a]

Fluid	Critical Temperature [°C]	Critical pressure [bar]
Carbon dioxide	31.1	73.8
Ethane	32.4	48.8
Ethylene	9.3	50.4
Propane	96.8	42.5
Nitrous dioxide	36.6	72.4
Methanol	240.1	80.9
Ammonia	132.4	113.5
Water	374.4	221.2
Trichlorofluoro-methane	198.1	44.1

[a] values taken from Reid et al. 1987.

Supercritical water has a high critical temperature of 374.4°C. Because of its high critical temperature and ability to dissolve oxygen it is able to maintain a flame-less combustion, and hence is currently being tested for combustion of organic toxic waste and destruction of chemical weapons (Bradley 1994).

1.2.2 Physical Properties of Supercritical Fluids

Supercritical fluids begin to exhibit significant solvent strength when compressed to liquid-like densities. As seen from Figure 1.2 the density varies greatly with pressure in the critical region and consequently the same holds for the solvent power of the fluid. The Figure shows distinctly the sharp increase in density in the critical region and this unique behaviour enables scientists to tailor the solvent power of supercritical fluids to achieve the required separation.

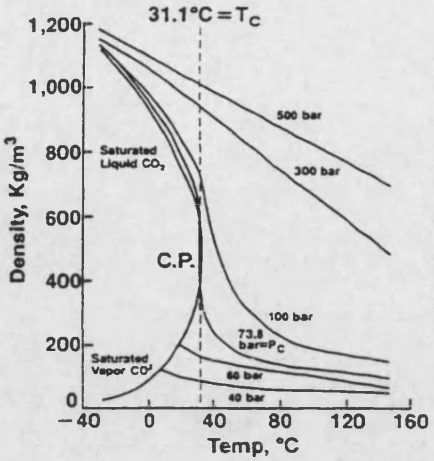


Figure 1.2 Variation of the reduced density ρ_R of a pure component in the vicinity of its critical point (Lira 1987).

As seen from Table 1.2 the densities of supercritical fluids reach almost the values of liquids, however when comparing the viscosities and diffusivities of supercritical fluids, the viscosities are almost gas-like (Schneider 1978, 1993).

Table 1.2 **Diffusivities D_{12}, densities ρ and viscosities η of gases, supercritical fluids and liquids**

	D_{12} [$cm^2\ s^{-1}$]	ρ [$g\ cm^{-3}$]	η [$g\ cm^{-1}\ s^{-1}$]
Gas	10^{-1}	10^{-3}	10^{-4}
Supercritical fluid	10^{-3}	0.2-0.8	10^{-4}
Liquid	10^{-6}	1	10^{-2}

Although the viscosity increases considerably with pressure it is still an order of magnitude lower than that of liquids (Filippi 1980 cited in McHugh and Krukonis 1994). Another advantage of supercritical fluid over liquids is the very low surface tension which enables fast penetration of the extraction material.

The frequently cited statement that the gas-like transport properties improve the rates of transport for solutes in supercritical fluid media, and hence result in faster extraction, is often misinterpreted. It is true that the rates of transport are enhanced in supercritical fluid compared to that in gases, however a faster extraction is only achieved if the rate-limiting step is the transfer of an analyte

from the surface of a solid phase to the supercritical phase. It does not hold true, if the analyte has to diffuse through a liquid phase as this imposes the rate-limiting step (McHugh and Krukonis 1994).

These physico-chemical properties in addition to the controllable solvent strength make supercritical fluids an almost ideal extraction solvent and encouraged many researchers to see SCF technology as a panacea for many problems.

1.3 Fluid Mixtures

The most commonly used supercritical fluids are relatively non-polar except for ammonia. In order to extend the use of supercritical fluid technology it is necessary either to use more polar fluids or to add a modifier (cosolvent, entrainer) to the fluid to enhance its solvent power. The more polar fluids have the drawback of possessing higher critical temperatures and pressures and cannot be used to extract or separate thermo-labile compounds. The reason for the higher critical temperatures of polar fluids is due to their increased attractive dipolar interactions requiring more energy to vaporise the polar fluid. However, since dipolar interactions are inversely proportional to temperature, this causes a concomitant decrease in the fluids ability for dipolar interactions (McHugh and Krukonis 1994).

The addition of a modifier complicates the system and makes potential applications more expensive. This disadvantage must be compensated by the advantageous properties of a fluid mixture. As the solubility of polar compounds is enhanced in modified fluids, lower pressures can be applied compared to pure fluids which presents a considerable energy saving in industrial applications. The solubility in the fluid mixtures often becomes more temperature dependent, as demonstrated in the separation of free fatty acids

from palm oil enabling fractionation just by changing temperature (Brunner 1982).

By careful selection of a cosolvent or modifier, engineers can therefore fine-tune the properties of a fluid to perform very precise separations or reactions.

1.3.1 Phase Behaviour of Mixtures

Fluid mixtures behave differently in the critical region and show altered phase behaviour. The necessity to know the phase behaviour of a mixture is twofold. Firstly, since most of the applied supercritical fluids are relatively non-polar, it is necessary to add modifiers to the fluid. These fluids are then used in supercritical extraction (SFE) and chromatography (SFC) and it is pertinent for reproducible results to be obtained to avoid phase separation in pumps, injectors, transfer lines, columns and detectors. In order to choose the appropriate conditions it is necessary to be aware of the critical parameters of the mixed fluid. Conversely, Clifford (1992) suggested that two-phase extraction might be advantageous in as far as it might cause agitation of the matrix and so enhance the extraction efficiency. Furthermore, when a supercritical fluid is used for the extraction of a certain compound, a binary mixture is formed. If the phase behaviour of this newly formed phase can be calculated, then in turn the solubility of the compound in the mixture can be deduced from equation of states instead of conducting numerous solubility measurements which require special equipment and are extremely time-consuming.

Van Konyenberg (1968) conducted an extensive study on critical lines and phase equilibria in binary van der Waals mixtures. The van der Waals equation of state was used in conjunction with a quadratic and linear mixing rule for the van der Waals constants a_m and b_m respectively, to determine the phase behaviour. The critical locus of the mixtures in the pressure-temperature composition space was calculated by solving a set of equations with the aid of a

computer. These calculations enabled the behaviour of mixtures to be categorised in Type I-V.

This thesis deals exclusively with the use of carbon dioxide and fluid mixtures produced with CO_2 and commonly used modifiers. Since these mixtures behave almost exclusively according to Type I, only this phase behaviour will be discussed.

The main characteristic of Type I phase behaviour is the continuous locus of the critical line which occurs when the mixture components are of similar molecular diameter or size and possess similar interaction strengths, hence there is only a small difference in the critical volume or temperature of the pure components (Rowlinson and Swinton 1982). When phase diagrams are used, it is essential to consider the phase rule, which was deduced by Gibbs (cited in Atkins 1990). It states the number of intensive variables that must be set to describe a phase behaviour completely.

$$F = C - P + 2 \qquad \text{eq. (1.1)}$$

F stands for the degrees of freedom, C for the components (2 in a binary mixture) and P for the number of phases present. In a binary system with 2 phases in equilibrium there are 2 degrees of freedom, meaning that two variables can be freely chosen (e. g. pressure and composition) and the temperature is dependent on the other two variables.

The vapour-liquid critical points of mixtures with different compositions lie between the critical points (CP) of the pure modifier and CO_2. Figure 1.3 shows the three most common critical loci and more variations of Type I behaviour are presented in the exceptionally valuable review of fluid phase equilibria by Page et al. (1992).

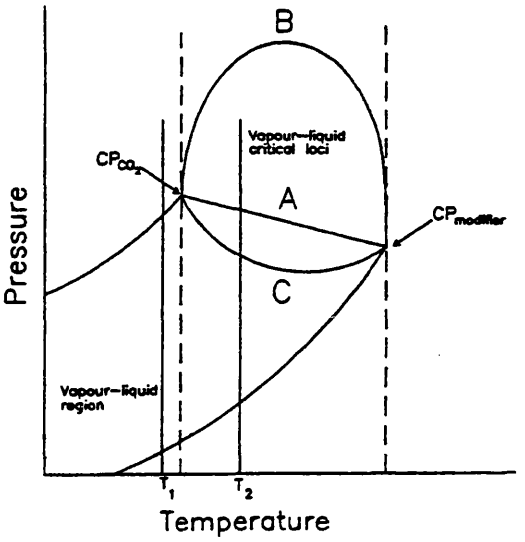


Figure 1.3 Different critical loci of Type I phase behaviour presented in P-T plot.

The critical loci in Figure 1.3 represents the critical points for different concentrations of modifier in mixtures. The line connecting the vapour-liquid curves of CO_2 and the pure modifier is called the vapour-liquid critical locus. The straight line A, connecting the critical points of CO_2 and the modifier, indicates almost ideal behaviour of binary mixtures according to Raoult's law due to components having very similar critical properties. An example for this ideal behaviour is observed for a mixture of CO_2 and N_2O (Rowlinson and Swinton 1982). When the vapour-liquid locus is moved upwards as in line B (Figure 1.3), then the mixture experiences a negative deviation from Raults` law. This is the most common phase behaviour and occurs in mixtures with similar critical pressures, but somewhat different critical volumes or temperatures like the behaviour exhibited by a mixture of CO_2 and MeOH. A downward curve in the critical locus indicates a large positive deviation from Raoults' law and can be observed in mixtures of polar and non-polar compounds.

Below the critical locus phase separation takes place, therefore, this region should be avoided while chromatographic separations are conducted. A single homogeneous phase exists above the critical locus.

In order to establish the concentrations at these critical points of the mixture in a 2-dimensional diagram, the temperature or pressure must be kept constant. If the temperature is kept constant and the case at $T_1$is considered in Figure 1.3, in which the temperature is chosen to be below the critical of the more volatile compound, the vertical line represents constant temperature. The line crosses both vapour pressure curves and the vapour pressures of the pure components are obtained. Figure 1.4, where x indicates the concentration of the lighter compound, shows the usual vapour-liquid envelope for binary mixtures. At low pressure there exits only one vapour phase over all concentrations.

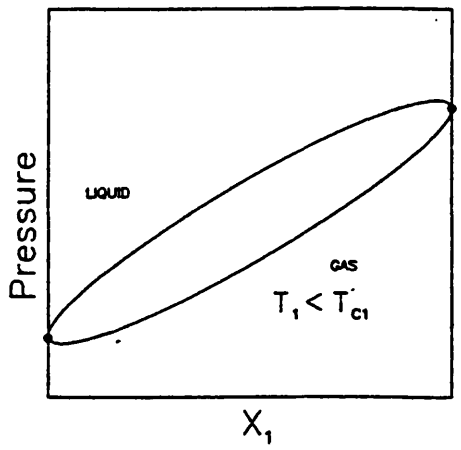


Figure 1.4 P-x diagram for a Type I mixture at a temperature T_1 below T_c of the lighter component.

Increasing the pressure of the mixtures causes the dew point curve to be intersected and the concentration of the component x can be determined by a horizontal tie line h. The point, at which the horizontal tie line crosses the dew point curve, gives the concentration of the lighter compound in the vapour phase and the other intersection point in the liquid phase. If the pressure is increased further, the amount of liquid increases while the amount of vapour reduces until finally all bubbles of vapour disappear. The locus that separates the two-phase vapour-liquid region from the single liquid region is called the

bubble point curve. If we consider a case in which the temperature (T_2) is above the critical point of the lighter compound, the vertical line in Figure 1.3 does not intersect the vapour pressure curve of the lighter compound anymore. However the intersection with the curve of the heavier compound again gives the point at the pressure axis in the P-x diagram in Figure 1.5. Thus, the vapour-liquid envelope at this temperature does not touch the pressure axis at the right-hand side since the vapour-pressure curve of the pure component was never crossed at this temperature. The critical point (CP) marks the intersection of the vertical line in Figure 1.3 with the vapour critical locus. Since this point is transferred in the P-x plot, the concentration of the modifier at this point is now known. All the parameters (pressure, temperature) necessary for obtaining a single supercritical fluid at the given temperature concentration are now known.

As seen from Figure 1.5 on the right-hand pressure axis the vapour pressure of the heavier component rises with increasing temperature which can also be observed in Figure 1.3 in the vapour pressure curve. When all temperatures, between T_c's of CO_2 and the modifier, are analysed in this way, then it is possible to construct a P-T-x diagram.

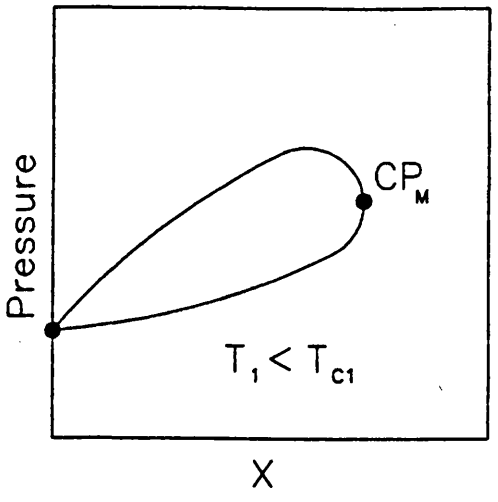


Figure 1.5 P-x diagram for a Type I mixture at a temperature T_2 above T_c of the lighter component.

1.3.2 Determination of Critical Loci

Critical loci are normally determined by experiments using light scattering to study phase behaviour (Page et al. 1991a, 1994). The CO_2-Methanol (MeOH) mixtures were prepared by introducing a pre-weighed amount of MeOH into an evacuated cylinder and adding CO_2. The contents were then mixed by agitation and transferred into a special flow cell, in which pressurisation took place until the critical mixture point was exceeded and the system was subsequently decompressed at a rate of 5atm/min. When a phase transition from a supercritical fluid of a liquid phase occurred, the intensity of light increased abruptly. The cell was weighed after the experiment in order to avoid alignment disruption and three-dimensional P-T-x diagrams could be constructed.

Ziegler et al. (1995) used an elegant chromatographic method for determining the vapour critical loci for binary mixtures. The critical pressure was obtained by injecting modifiers with a fixed injection loop at various pressures at a constant temperature and subsequent evaluation of the resulting chromatograms. If the binary mixture was below the critical point (CP_M) of the mixture, the peak shape was rectangular. Above the CP_M, skewed Gaussian peaks were obtained. However as the study was carried out using a given amount of modifier which was not quantified, it was not possible to plot a P-T diagram which allowed pinpointing the concentration of each critical parameter as in the diagram in Figure 1.3. When all three parameters (temperature, pressure and composition) are known, a three-dimensional phase diagram can be constructed. However, this is not mandatory as the phase rule states that only 2 independent variables are necessary to determine the phase behaviour.

Most other studies of phase equilibria involve the visual observation of dew point, bubble point or critical opalescence and subsequent analysis of the phase composition allows three-dimensional P-T-x diagrams to be constructed. Depending on the temperature, different phenomena can be observed in a view cell when a Type I binary fluid mixture is decompressed (Figure 1.6). If the

temperature is above the mixture critical point and decompression from D to A takes places, the dew point line is crossed and the amount of liquid initially increases. Further decompression decreases the amount of liquid and when the dew point curve is crossed at A, liquid is no longer present. This phenomenon is called retrograde condensation, since there is no heat involved as would normally be observed for condensation.

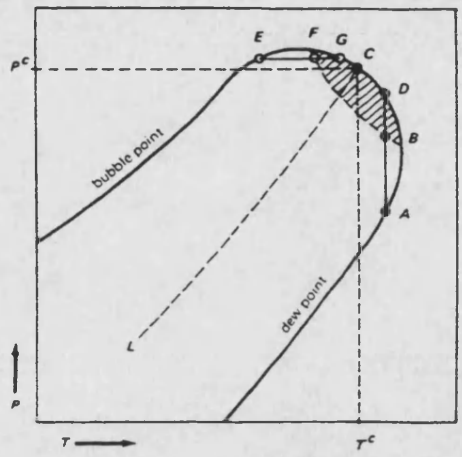


Figure 1.6 Retrograde condensation in a system of constant composition.

Critical opalescence is observed when decompression takes place at T_c of the mixture. The mixture has a characteristic bluish colour if light is reflected through the solution and a characteristic reddish orange colour if light is transmitted through the solution (Travers and Ushers 1906 cited in Page et al. 1992). This can be explained by the infinite compressibility of the mixture at the critical point, resulting in an enormous density change during decompression. Since density is associated with the refractive index the change in density causes this colour appearance. If the temperature at decompression is below T_c of the mixture, the bubble point curve is crossed and one observes the formation of bubbles in the single liquid phase. Further decompression will eventually result in crossing the dew point curve and only vapour will be present.

Brunner et al. (1985, 1987) determined the isothermal phase equilibria in binary mixtures consisting of methanol plus hydrogen, nitrogen, methane or carbon monoxide or carbon dioxide.

McHugh and Krukonis (1994) outlined instrumental techniques for determining phase border curves. Page et al. (1992) reviewed all the existing phase equilibria of CO_2 and mixtures with the most commonly applied modifiers and highlighted the need for further investigation of phase behaviour of binary or ternary mixtures in order to produce reliable results in SCF technology.

1.3.3 Calculation of Critical Loci

Measurements of critical loci require special equipment which is not available in many laboratories, therefore reliable estimation methods for mixture critical fluids would be a valuable asset for the prediction of the CP_M of binary or ternary fluid mixtures to avoid phase separation.

Since the critical parameters of pure components are required for the calculation of the mixture's critical parameters it is essential to have accurate values so as not to introduce errors in this first step. There are a plethora of publications concerned with critical parameters (Reid et al. 1987, Kreglewski 1984), however Page et al. (1992) found considerable differences between much of the published data.

Estimation methods for the critical constants of pure components are mainly group contribution methods. They are based on the fact that all macroscopic properties are related to structure, which determines the magnitude and predominant type of the intermolecular forces (Reid et al. 1987). Page et al. (1991b, 1992) critically evaluated numerous methods of estimating T_c, P_c and V_c.

They also recommended estimation methods of critical parameters for specific chemical classes of compounds with which the best and most reliable results were obtained.

For the prediction of vapour-liquid loci, Page et al. (1991b, 1992) reviewed the most common and relatively simple estimation methods for the critical parameters for mixtures. Generally, the approach of Chueh and Prausnitz was used for the critical pressure and temperature of mixtures, the approaches of Kreglewski and Kay and that of Li are applied for the critical temperature. Reid et al. (1987) presented examples of the most common approaches to calculate the critical parameters of mixtures: T_c estimates by a method of Chueh-Prausnitz, Li and Kreglewski and Kay, V_c by a method of Schick and Prausnitz and P_c by the approach of Kreglewski and Kay. Additional methods of estimating critical parameters were explained by Danner and Daubert (1983). All these equations are generally for non-polar hydrocarbon mixtures and are based on Type I phase behaviour. Thus, it is hardly surprising that Page et al. (1991b, 1992) found great differences between measured vapour-liquid critical loci and estimations using the above methods.

For the estimation of the true critical temperature and pressure Reid et al. (1987) recommended a pressure explicit equation of state (EOS) being either the Soave-Redlich-Kwong or the Peng-Robinson equation. However, neither equations gave good estimates for the critical volume. Conversely, McHugh and Krukonis (1994) found good correlation between experimental data and calculations using the Peng-Robinson EOS. The Peng-Robinson EOS with two fitted parameters, determined by experimental data correlation, enabled fewer experiments to be conducted and hence was not as time-consuming.

The calculation of phase equilibria is exhaustively covered in a book edited by Sandler (1994), however these calculations are mainly directed to chemical engineers for whom the calculation of vapour-liquid and liquid-liquid equilibria are important for process design.

1.4 Solubility

Hannay and Hogarth (1880) were the first to discover increased solubility of solid compounds in compressed gasses at pressures at which their density approaches the densities of liquids occurring near or beyond the critical point.

Hannay (1880) gave the following explanation:

> "The liquid condition of fluids has very little to do with their solvent power, but only indicates molecular closeness. Should this closeness be attained by external pressure instead of internal attraction, the result is that the same or even greater solvent power is obtained ....."

The above statement may be misleading, in so far as the conclusion from this might be that by compressing gases to very high pressures it is possible to dissolve the majority of compounds in compressed gases.

The most extensive study of solubility in CO_2 was investigated by Francis (1954) using 261 substances. Francis observed that CO_2 was a poor solvent when used at higher concentrations of 60 to 90%, however showed a strong mixing action at 40% when it acts as a dissolved gas. Dandge et al. (1985) related the solubility of organic compounds to their structure. The compounds were separated into chemical classes and the influence of variation in structure was discussed.

1.4.1 Parameters Influencing Solubility

Whether a given solute dissolves in a SCF depends on two factors. Firstly, a solid is soluble in a fluid if the free volume difference between solute and solvent are negligible. This gives an estimation of the compressibility of the solute and solvent and hence is an indication of whether the molecules in solution are close enough to interact (McHugh and Krukonis 1994). Secondly,

intermolecular forces between solvent-solvent, solvent-solute and solute-solute pairs in solution are important in determining whether a solute is soluble in a solvent.

Johnston et al. (1982) stated that solubility is related primarily to the liquid-like density which promotes strong attractive forces by increasing the probability of interactions, however it does not influence the basic nature of the forces. The reason for density to be considered as being the prime factor influencing solubility was that most solubility graphs depicting solubility versus density looked like graphs of density versus pressure. However, these systems were mainly non-polar so that dispersion interaction between the solutes' molecules was comparable to the interaction between the solvent molecules, thus making density the prime factor influencing solubility.

If a solute is soluble in a SCF, the solubility is influenced by density, pressure and temperature. An increase in density at constant temperature leads to enhanced solubility and an increase in temperature at constant density also enhances the solubility due to an increase in the solute's vapour pressure. Increasing the temperature at constant pressure, a slightly more complicated solubility behaviour is observed for a non-volatile compound. If the temperature was increased at low pressure, a decrease in solubility would be noted, whereas at higher pressures enhanced solubility is observed. Figure 1.7 shows solubility plotted versus pressure at different temperatures and the above mentioned phenomena can be seen. At higher pressure the solvent density is influenced to a lesser extent by temperature and hence the enhanced solubility at higher temperatures stems from an increase in vapour pressure.

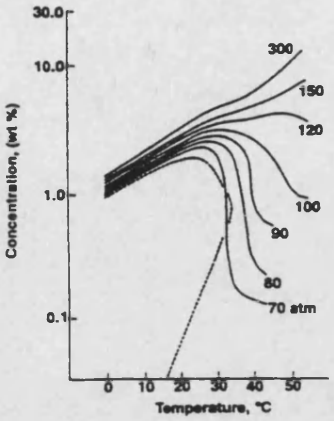


Figure 1.7 Solubility isotherms for naphthalene in CO_2.

Zosel (1978) explained these two processes in terms of distillation and extraction mechanisms taking place concomitantly. The distillation effect is the vaporisation of the solute in which the vapour pressure is the dominant factor, whereas intermolecular forces govern the extraction process.

Since intermolecular forces ultimately determine the degree of solubility it is necessary to understand the intermolecular forces prevailing between solute and solvent molecules in a mixture.

The forces between solute-solute, solute-solvent and solvent-solvent molecules can be separated into physical and chemical interaction. Chemical interactions include hydrogen-bonding, acid-base interactions and electron-donor complexing and are difficult to quantify due to their complexity. Nevertheless, it is possible to explain their impact on solubility using co-solvent studies.

There are several possible physical interaction, which can be separated into dispersion interactions, permanent dipoles, quadrupoles moments, quadrupole-dipole interactions and quadrupoles/dipole-induced dipole (McHugh and Krukonis 1994).

In order for a solvent to be dissolved by a fluid it is important that the strength of the solvent-solvent interactions and solute-solute interactions are comparable with the solvent-solute interactions as otherwise it is unlikely for the solute to be soluble in the solvent.

However, at very high pressures or consequently very high density, the repulsive forces become large and hence the solid solubilities decrease with increasing density/pressure at very high pressures (Lira 1988 cited in McHugh and Krukonis 1994).

To explain solubility various groups have used spectroscopic studies to elucidate molecular interactions. The Taft-Kamlet linear solvation energy relationship is used to characterise solvents or fluids according to their polarity, hydrogen donor and acceptor ability (Kamlet et al. 1983). Hyatt (1984) used visible spectroscopy of solvatochromic dyes, infrared spectroscopy of ketones and pyrrole and the exo:endo ratio of a Diels-Alder reaction in CO_2 as indicators of solvent polarity. The bathochromic shift of an azo dye was studied and the shift caused by supercritical CO_2 correlated to Taft-Kamlet π^* scale of solvent dipolarity and polarisability. They concluded that liquid and supercritical CO_2 have dipolarities close to those of hydrocarbons, but have polarisabilities even lower than those of fluorocarbon solvents. This statement was confirmed with the IR study of acetone and cyclohexanone and the Diels-Alder reaction of cyclopentadiene. In the IR study where pyrrole was disolved in CO_2, did CO_2 behave like a solvent in the polarity range of ether to ethyl acetate range and it was concluded that CO_2 may have the hydrogen bond basicity of ether-ethyl acetate. Lee et al. (1990) classified CO_2 as Lewis base able to have acid-base, induced dipole and quadrupole interactions. Schneider (1992), contrary to many other citations (McHugh and Krukonis 1994), summarised CO_2 as having only negligible quadrupolar interaction and hence its solubility strength is based only on dispersion interactions.

Additional spectroscopic studies resulted in the concept of clustering, where solvent molecules are tightly packed around the solute, when the solvent was very compressible near the critical point and explained increased solubility at this point with the clustering phenomena (Yonker et al. 1986). However, the concept of a static stable aggregate of solvent molecules clustered around a solute molecule has been changed to a concept where the enhanced local properties are explained as a dynamic interchange of an excess of solvent molecules entering and leaving the region in the immediate proximity of the solute molecule, due to attractive solvent-solute interactions (Shaw et al. 1991).

1.4.2 Calculation of Solubility using EOS

The measurement of solubility is extremely time-consuming and therefore the use of EOS with empirical constants, which are determined by a limited number of solubility experiments, subsequently allows the prediction of solubility at different temperatures and pressures from the derived equations.

There are several models of supercritical phase equilibria which can be used to calculate solubility of solids in SCF. Most commonly, the SCF phase is treated as a dense gas and an EOS is used to calculate the fugacity coefficient, ϕ_i, of the component i in the SCF phase:

$$f_i^{SCF} = y_i \, \phi_i \, P \qquad \text{eq. (1.2)}$$

where f_i^{SCF} is the fugacity, y_i is the mole fraction of component i in the SCF phase and P is the pressure. The fugacity coefficient is a measure of the difference between ideal gas behaviour, where no interaction between molecules is taking place and real gas behaviour. Since the solid phase is in equilibrium with the supercritical phase the fugacity of compound i in the SCF phase must also be equal to the one in the solid phase. The fugacity of compound i in the solid phase is given by

$$f_i^s = P_s^{sat} \exp\left(\frac{v_2^s(P - P_2^{sat})}{RT}\right) \qquad \text{eq. (1.3)}$$

where P_2^{sat} is the sublimation pressure and v_2^s is the molar volume of the solid. The following assumptions have been made: a) the fluid-phase components do not dissolve in the solid, b) the molar volume of the solid is independent of pressure and that the pure saturated solid at temperature T is ideal which means its fugacity is unity.

Equation 1.4 is obtained by combining Equation 1.2 and 1.3 and gives the solubility of the solid in the SCF phase (y_2^s):

$$y_2^s = \frac{P_2^{sat} \exp\left(\frac{v_2^s (P - P_2^{sat})}{RT}\right)}{\phi_2 P} \qquad \text{eq. (1.4)}$$

The ratio of the actual solubility to the solubility in an ideal gas ($y_{2,ideal} = P_2^{sat} / P$) is the enhancement factor. In order to predict y_2 accurately, not only are accurate EOSs necessary, but accurate vapour pressure data are required. However, since the fugacity coefficient in the SCF is not known, EOSs are used to calculated initially the fugacity coefficient from experimentally obtained solubility data.

Several different types of EOS can be used to calculate the fugacity coefficient and can be divided into the following main families:

- the van der Waals family of cubic equation of state
- the virial family of equation of state

Only the main and most common EOS are listed here, for more details about all currently used EOS, there is an extensive discussion in the book of Sandler (1994).

Cubic EOS, which are the most widely used, are normally used for the calculation of the equilibrium concentrations of a vapour and a liquid of a pure component. They can be extended to two components when mixing rules are applied which are crucial in determining the quality of the model. Since there is a plethora of cubic EOSs the procedure to calculate the fugacity coefficient is limited here to the description of the simplest EOS, the van der Waals equation, and since most of the other equations are all derived from the basic van der Waals equation it should be adequate to demonstrate the basic principle.

One of the earliest cubic EOS is the van der Waals equation and it can be applied to mixtures using the van der Waals one-fluid mixing rule,

$$a = \sum\sum x_i x_j a_{ij} \qquad b = \sum\sum x_i x_j b_{ij} \qquad \text{eq. (1.5)}$$

where a and b are van der Waals parameters. In addition, combining rules are needed for the estimation of the parameters a_{ij} and b_{ij}. The usual combining rules are

$$a_{ij} = \sqrt{a_{ii} a_{jj}}\,(1 - k_{ij}) \qquad b_{ij} = \frac{1}{2}(b_{ii} + b_{jj})(1 - l_{ij}) \qquad \text{eq. (1.6)}$$

where k_{ij} and l_{ij} are the binary interaction parameters obtained by regression of the experimentally measured solubility data. The van der Waals EOS can predict almost all types of phase behaviour qualitatively as presented by van Konyenberg, but lacked accurate quantification (Ekart et al. 1991). It is also noteworthy that EOS should only be used in cases in which the interaction of solute and solvent are restricted to dispersion forces and since the mixing rules assume random mixing no directional forces such as hydrogen-bonding should be present when mixing rules for apolar systems are applied.

Using equation 1.4 it is possible to show that there are two competing effects that determine the solubility of a solid at constant temperature in SCF (Lira 1988 cited in McHugh and Krukonis 1994). The solubility increases with increasing pressure since the fugacity coefficient, ϕ_i^{SCF}, decreases far more rapidly than pressure or the exponential term increases. On the other hand ϕ_i^{SCF} increases at very high pressures where the repulsive forces dominate, thus lowering solubility.

Many researchers have correlated solubility data for solid nonpolar hydrocarbons such as ethane, ethylene and CO_2 with the Soave-Redlich-Kwong or Peng-Robinson equation of state by including a binary interaction parameter, k_{ij}, to fit the data best. Since k_{ij} is usually temperature or density dependent, it is necessary to introduce more parameters. Another possibility is using estimation methods for predicting k_{ij} (Sandler 1994).

Kurnik et al. (1981) used both the Soave and the Peng-Robinson modification of the Redlich-Kwong EOS for the regression of the binary interaction parameter and observed equally good estimates for both approaches. They measured the solubility of several nonpolar compounds and the slightly polar benzoic acid using supercritical CO_2 and ethylene. They concluded that all data was well correlated, however McHugh and Krukonis (1994) observed that the solubility data of benzoic acid is rather poorly fitted since a temperature dependence of the binary interaction can be observed even in a very narrow temperature interval of 20°C. Schmitt and Reid (1986) investigated the solubility of organic solids in various SCFs. They modified the existing Peng-Robinson equation since it did not correlate experimental solubility data very well over a wide range of pressures, even when the binary interaction parameter was optimised. The Peng-Robinson equation was initially derived for a liquid-vapour system and its application to solids requires that the solid phase is regarded as a liquid. They derived a slightly altered equation possessing two interaction parameters which were obtained using non-linear regression of the experimental data. The two

binary interaction parameters were again slightly temperature dependent, however the ratio between the two parameters stayed constant. Moreover, the binary interaction parameters were different for a given solute when different SCFs were used for the solubility measurements which reflected the difference in the ability of the different SCFs to dissolve a given solute. The study illustrated that chemical effects were important for solubility and the broad concept of like dissolves in like can also be applied to SCF technology. Bartle et al. (1992) derived a correlation for the binary interaction parameter in the Peng-Robinson equation with a group of physical parameters of SCFs, for example CO_2 and several others solutes. An almost linear relationship between these and the binary interaction parameter was found for a variety of compounds after the introduction of a factor for alcohols and carboxylic acids. When no solubility data is available, the binary interaction parameter can be deducted from a linear relationship of the binary interaction parameter with the difference of acentric factors of the compound and the one of the SCF. However, they stressed that these equations are preliminary and tentative, and need further study.

As Johnston et al. (1989) pointed out, no single model for describing the complex phase behaviour will work for all situations (Johnston et al. 1989 and Wong et al. 1985). Once the understanding of molecular interactions in SCF becomes more accurate, better models can be deduced. Thus, so far EOS offer the best compromise between accuracy and ease of application.

1.4.3 Predictive Methods of Estimating Solubility

The calculation using EOS required the data of experimentally determined solubility data and additional physicochemical data to characterise solute-solvent interactions. Their application has only been evaluated for fairly simple systems and have not yet found wide acceptance.

Giddings et al. (1968) aimed to find a common mathematical framework to correlate the solubility in dense gases with liquids and to enable scientists to predict pressures needed to achieve the required solubilisation. Giddings distinguished between a state effect and chemical effect to describe the solvent power. The chemical effect is unique to each chemical class and incorporated a compound's polarity, acid-base property and hydrogen bonding tendency. Since normal liquid solubilisation depends exclusively on the chemical effect, it was apparent that there was an additional degree of freedom when using compressed gases. They compared Snyder's elution power value, ε^0, with Hildebrand's solubility parameter and found a reasonable correlation and thus hoped to correlate the elution power of compressed gases with the help of the solubility parameter. They derived the following widely applied relationship by assuming that a) the solubility parameter includes both state and chemical effect and b) the van der Waals equation is applicable even at high densities.

$$\delta = 1.25\, P_c^{1/2} [\rho_r / \rho_{r,liquid}] \qquad \text{eq. (1.7)}$$

where δ is the solubility parameter, P_c is the critical pressure of the liquid or gas, ρ_r is the reduced density, and $\rho_{r,liquid}$ is the reduced density of the gas or liquid at its normal boiling point. The solubility parameter δ was defined by Hildebrand et al. (1970) and it is the square root of the cohesive energy density which is a measure of attraction and represents the energy required to remove a molecule from its own liquid. It can be calculated from the expression

$$\delta = (\Delta U / V)^{1/2} \qquad \text{eq. (1.8)}$$

where ΔU is the internal energy change during vaporisation and V is the liquid molar volume.

Solubility parameters were calculated using equation 1.7 for compressed gases and the solubility parameter of CO_2 was estimated to be comparable to liquid 2-propanol when SC-CO_2 approaches the density of a liquid requiring a

pressure of about 1000atm. Giddings stressed that this derivation stretches the concept of regular solution to its limits, since it should only be applied to systems where pure dispersive interactions are present. Equation 1.7 is further derived on the assumption that dense gases behave according to the van der Waals equation which is known to be only valid for qualitative predictions.

King (1989) extended the use of the equation derived by Giddings by combining it with the Flory-Huggins concept for the calculation of the threshold pressure for solute solubility. The threshold pressure can be estimated by calculating the intersection of the Flory interaction parameter in equation 1.9 and the Flory critical parameter. Giddings et al. (1969) defined the threshold pressure, which is the pressure at which the solute begins to dissolve in the SCF.

The Flory-Huggins concept was developed for the regular solution theory of high-molecular weight compounds in liquids (Hildebrand et al. 1970). These solutions showed deviations from Raoults' law, leading to the introduction of the Flory interaction parameter, χ, which depends exclusively on the intermolecular forces between solute and solvent. Both excess entropy and excess enthalpy contribute to χ

$$\chi = \chi_S + \chi_H = \chi_S + \overline{V}_1 (\delta_1 - \delta_2)^2 / RT \qquad \text{eq (1.9)}$$

where χ_S is the contribution from the excess entropy and χ_H is that from the excess enthalpy, $\overline{V}_1$ is the molar volume of the gas as a function of pressure, δ_1 is the solubility parameter of the gas as defined by equation 1.7 and δ_2 is the solubility parameter of the solute as a function of temperature.

The Flory critical parameter can be calculated with the following equation

$$\chi_c = (1 + x^{1/2})^2 / 2x \qquad \text{eq. (1.10)}$$

where x is $\overline{V}_2 / \overline{V}_1$ and $\overline{V}_2$ is the molar volume of the solute.

When both the Flory interaction parameter and the critical parameter reach the same value, then miscibility occurs.

Another useful parameter is the pressure at which maximum solubility is reached which can be calculated using equation 1.7 by assuming that maximum solubility is reached when the solubility parameter of the compressed gas is equal to the solubility parameter of the solute. If values of $\delta_{2,solute}$ are not available in the literature they can be estimated using the group contribution method of Fedors (1974) and their temperature dependency nomograms are used (Jayasri and Yaseen 1980).

The pressure region between the threshold pressure and the maximum pressure is called the fractionation pressure range (King 1989). King found good correlation between experimentally determined and calculated threshold pressures when the concept was applied to triglycerides. Additional calculations were conducted for the fractionation of DDT from lard, predicting a threshold pressure of 180atm. Experiments confirmed this result to be correct, since only a trace amount of DDT was extracted at 95atm in comparison to 75% at around 200atm. The usefulness of this approach in estimating threshold pressure and thus being able to define the fractionation pressure range when no chemical interaction are present was therefore demonstrated. Allada (1984) showed a generalised method for determining the solubility parameter of an SCF, but found that δ alone could not correlate solubilities of a given system in diverse solvents.

Indeed, efforts to apply regular solution theory to SCF equilibria required the application of semi-empirical modifications. Pang and McLaughlin (1985) were able to fit the solubility of several aromatics in CO_2 and ethylene as a function of the solubility parameter, but four adjustable parameters were necessary. Mitra and Wilson (1991) used a statistical analysis package to perform multiple regression for the correlation of solubility and process conditions. Initially the solubility was evaluated as a function of density and temperature since

solubility is considered to correlate better with density than with pressure. An equation with three parameters was obtained

$$\ln(s) = a\,d + b\,T + c \qquad \text{eq. (1.11)}$$

where s is the solubility of the compound, *a* reflects the solubility change with density at constant temperature and *b* the solubility change with temperature at constant density. The slope is given by -a/b and depends on the solute-solvent interaction as well as the volatility of the solute. The slope varied for different systems and was an indication of the relative dependence of solubility on temperature and density. The equation was tested on solvents and fluids of different properties over a wide range of polarities, temperatures and pressures, which proved the validity of the equation. It showed that solubility increases with increasing temperature at constant density. In order to correlate solubility with pressure and temperature it was necessary to introduce 5 parameters. The simulated solubility showed the expected behaviour. The solubility decreased at low pressures with increasing temperature, but increased with increasing temperatures at elevated pressures. In order to derive the five parameters at least five solubility experiments at different temperatures and pressures are necessary.

1.4.5 Cosolvent Effects on Solubility

Adding modifiers not only increases solubilities of moderate polar to polar compounds in nonpolar SCFs, hence lower pressures can be used for extraction processes, making the process more economically viable, but it also can enhance the selectivity through specific interactions to enhance the fractionation of compounds.

Spectroscopic studies were conducted to elucidate the cause of the increase in solubility, since many researchers claimed it was due to density increase in the

fluid mixture when adding modifier. Yonker and Smith (1988) investigated the solvatochromic shift for binary supercritical fluid mixtures (CO_2 + 2-propanol) as a function of modifier mole fraction, temperature and pressure. The theory behind solvatochromic shifts is that the nearest-neighbour solvent shell about a solute, influences the stabilisation of the excited-state dipole of the solute molecule and by measuring this effect by wavelength shift of absorption maxima, the local composition of pure and binary systems can be evaluated. Yonker and Smith concluded that the observed enrichment of 2-propanol around the solute was caused by specific interactions, in particular hydrogen-bonding interactions. The local composition of 2-propanol was highest in conditions at which the mixture had the highest compressibility. The wavelength shift versus pressure isotherms followed trends observed for density versus pressure isotherms, hence it may have been concluded that the shift was solely caused by changes in fluid density. However, by evaluating the solvatochromic shift of the mixture in relation to one of the pure fluid, it was shown that 2-propanol was present at higher concentrations around the solute than in the bulk composition of the mixture. If the fluid was below the critical temperature, thus subcritical, the solvatochromic shift varied only modestly with respect to pressure since the fluid was less compressible. The same held true at high pressures where a further increase in pressure again caused only a moderate increase in the solvatochromic shift. This was due to a reduction in the effect of the attractive forces on the structure of the fluid as the molecules become less mobile. The enrichment of 2-propanol at higher temperatures was less than at lower temperatures due to the fact chemical interactions normally decrease with increasing temperature. Chemical forces normally decrease with temperature, since they are dependent on the alignment of molecules in solution (McHugh and Krukonis 1994).

The presence of hydrogen-bonding between methanol and CO_2 was confirmed by Fourier transform infrared spectroscopy in a study by Fulton et al. (1991). Five indicators which strongly suggested the existence of specific

interaction between CO_2 and methanol were discussed and the likely source of hydrogen bonding was identified, namely the attraction between the relatively large quadropole of CO_2 and the large dipole of the hydroxyl group in methanol.

Dobbs et al. (1987) conducted a thorough study into the effect of cosolvents on solubility taking the density effect into account. They observed increased solubilities of solids when specific interactions between solute and cosolvent were present. The specific interactions were separated into dispersion, orientation and hydrogen bonding or acid-base forces. With the help of specific solubility parameters the internal energies of each interaction could be calculated and a binary interaction parameter for the solute and cosolvent was obtained. This interaction parameter was then incorporated into an EOS. The prediction, however, when using the final equation was only qualitative since polar systems are far more complex due to the complexity of polar forces.

Wong and Johnston (1986) investigated the ability to dissolve biomolecules in fluid mixtures and observed a rise in solubility of some sterols by up to two orders of magnitude when methanol was added due to hydrogen bonding. The selectivity was not improved when no specific complexes were formed due to the similar interactions present in all sterols. Increased selectivities without a decrease in yield were observed in a mixture of two solids using a fluid mixture when the two solutes had different polarities, since the cosolvent only interacted with the polar solid due to specific interactions (Dobbs and Johnston 1987).

Kim and Johnston (1987) tried to incorporate the clustering of cosolvent around the solute and the specific interaction into a new EOS, called Augmented van der Waals-density dependent local composition model. They quantified the local composition of different cosolvents around a probe by spectroscopic means and evaluated the differences between nonpolar, aprotic dipolar and two protic solvents. The decay in the local composition with pressure was larger for octane than for the polar cosolvents, which suggested that the solvation due to hydrogen bonding is less dependent on compressibility

than that due to nonpolar forces (Kim and Johnston 1987). Kim and Johnston used the new EOS to calculate local composition and found general agreement with the measured values. Furthermore, they tested the equation to predict solubilities in fluid mixtures using the method of Dobbs et al. (1987) to calculate the binary interaction parameter which incorporated the extent of specific interactions. They found a considerable improvement in predicting the effect of cosolvents on solubility more accurately than previous models since it included the local composition effect as well as specific interactions.

1.5 Supercritical Fluid Chromatography

The phenomenon of supercritical fluid behaviour was applied to chromatography by Klesper et al. (1962) for the separation of porphyrins using a home-built apparatus. Sie et al. (1966 cited in Lee and Markides 1990) named this kind of chromatography "supercritical fluid chromatography" to correspond with gas and liquid chromatography. Since the vapour pressure and hence temperature is the main control over retention in gas chromatography, temperature gradients are used to extend the usefulness of gas chromatography. Equally, the mobile phase composition is changed to control the retention in LC, thus Sie and Rijnders extended the theory to SFC and suggested the use of mobile phase pressure programming.

Jentoft and Gouw were the first to introduce mobile phase modifiers in 1970 and also used pressure programming to control retention in SFC (Jentoft and Gouw 1970).

Since its revival in the 1980's due to the availability of commercial instruments, SFC has been extensively studied and applied to a variety of applications (Smith et al. 1988). Extensive reviews of applications of supercritical fluid chromatography were published by Chester and Pinkston (1990), and Chester et al. (1992, 1994, 1996).

Supercritical CO_2 is the most commonly used fluid due to its moderate critical pressure (73.8bar) and its low critical temperature (31.1°C). Its low toxicity and reactivity in conjunction with its high purity at low cost is a major incentive to use SCF CO_2.

Generally, the mobile phase in SFC should consist of one single phase, since chromatography conducted in the two-phase region produces irreproducible retention times and causes a very noisy baseline (Page et al. 1991). The parameters necessary for a single phase SCF are described in Chapter 1.3.2.

1.5.1 Instrumental Requirements

The renaissance of SFC using packed columns is believed to have coincided with the introduction of the commercial SFC instrument by Hewlett-Packard (Taylor and Chang 1990). The number of publications using packed column instruments is increasing and in particular in their application to chiral separations (Chester et al. 1996). However, there is still no simple SFC instrument available for the novice (Chester et al. 1996), and that could be the cause for SFC being still a specialised technique in pharmaceutical and bioanalysis (Wilson et al. 1993).

The instrumentation as seen in Figure 1.8 for packed column supercritical fluid chromatography is generally comprised of a CO_2 cylinder, reciprocating pumps for the supply of CO_2 and modifier and a mixing device so as to produce homogeneous fluid mixtures.

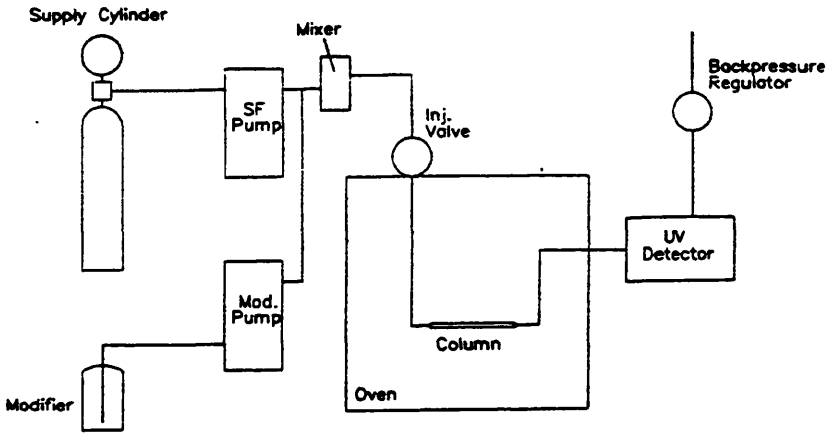


Figure 1.8 Simplified diagram of a basic packed column SFC system.

An injection valve allows the injection of samples onto a column, which is held in a precision temperature controlled oven, a detector of choice and most importantly a backpressure regulator which maintains the system at a predetermined pressure above the critical pressure.

Fluid supply: the most common way of CO_2 withdrawal is from a cylinder equipped with a dip tube, which allows liquid withdrawal. Alternatively, if there is no dip tube, the cylinder is inverted. The CO_2 in the cylinder is mostly liquid with a gas headpressure, which forces the liquid through the tubing into the pump. Adding helium into the cylinder increases the headpressure, which enhances the liquid delivery to the pump, especially when syringe pumps are used. However Via et al. (1994) and Porter et al. (1987) noted that helium is soluble in CO_2 and caused changing retention times.

When mixed mobile phases are required for the elution of more polar compounds and when only one pump is available, premixed cylinders containing various concentrations of modifiers can be purchased. The headspace of the gas has a significantly different composition from the liquid, hence the

equilibrium concentration changes during the cylinder use. Via et al. (1994) and Schweighardt and Mathias (1993) observed modifier concentrations to change more than twice the original amount.

Pumps: Reciprocating pumps used in HPLC were initially converted by researchers for the use for SFC. Greibrokk et al. (1984, 1986) modified two different versions of Waters pumps by drilling cooling channels into the pump head and constructing a clamp-on cooling unit for the checkvalves, and a clamp-on heat exchanger for the pump heads and check-valves respectively. Other researchers modified HPLC pumps in a similar way and used various cooling liquids to avoid formation of gas bubbles in the pumps (Mourier et al. 1985, Simpson 1986, Smith and Sanagi 1990). Gere et al. (1982) and Saito and Takeuchi (1989) recommended cooling of the pump head to -5 C to counteract the high compressibility and low viscosity.

The addition of modifiers is normally achieved with a second reciprocating pump, followed by a mixer which usually consists of a T-piece, a HPLC column filled with stainless steel balls or a dynamic mixer to produce a homogeneous CO_2 and modifier mixture.

Restrictors: These have the important task of maintaining the system at a chosen pressure above the critical pressure, so that the fluid is dense enough to have considerable solvation power.

Two basic types of restrictors exist. The first class belong to the fixed restrictor type, which can be divided into linear, tapered or fused silica, integral, porous and pinched restrictors. The ideal restrictor should a) provide uniform, pulse-free flow over a variety of flow rates, b) be immune to plugging, c) be easily replaceable, c) transfer labile and non-volatile compounds quantitatively and e) result in a minimum of peak broadening (Vejrosta et al. 1993).

Fixed restrictors do not allow the flow rate to be chosen independently from the system pressure. In order to increase the pressure in a system, the flow rate

has to be increased, causing velocity gradients to be superimposed onto pressure gradients, unless the temperature was controlled (Berger and Toney 1989). Increasing flow rates with increasing density is detrimental to efficiency, as both the diffusion coefficient and linear velocity increase, thus increasing the resistance to mass transfer. Berger (1989a) further studied the mass flow effects of restrictors quantitatively and concluded that turbulent flow in the restrictor is desirable and this could have been utilised with an ideal pinhole restrictor. However, he also pointed out that at high pressure gradients charging effects dominated flow and efficiency.

One of the earliest restrictor was the linear restrictor which consisted of a very narrow (10-50 μm) fused silica capillary (Fjeldsted et al. 1983). In this restrictor the depressurisation of the fluid occurred over the whole length, which caused plugging problems since the solubility of the solids decreased rapidly. Berger (1989b) concluded that restrictors having laminar flow patterns show a more significant increase in linear velocity with pressure increase than restrictors with turbulent flow patterns.

This led to the development of tapered or drawn fused silica restrictors (Chester et al. 1985) and the integral restrictor by Guthrie and Schwartz (1986). Green and Bertsch (1988) looked at the potential use of pinched metal restrictors in comparison to glass and fused silica restrictors. They concluded that the metal restrictor would be easier to control thermally to overcome the cooling effect due to the adiabatic expansion, however it showed significant activity towards polar compounds. Porous frit restrictors were developed as an alternative since they are relatively rugged and do not plug easily, but they often discriminated against high molecular compounds (Markides et al. 1986). Vejrosta et al. (1993) developed a porous restrictor which can be easily and reproducibly replaced.

The other class of restrictors are the manual and automatic variable backpressure regulators. With manual restrictors it is not possible to run a

pressure program, however modifier gradients are possible. All variable restrictors have the advantage of maintaining constant flow rate even when pressure or modifier concentration is changed. This is more favourable with the resistance of mass transfer at higher densities. The most common types are spring-loaded pressure-relief valves, which are able to maintain a constant pressure at different flow rates and viscosities. Pressure and modifier programming with a modified self-adjusting valve and a dual pumping system was shown by Morrissey et al. (1991) to produce very reproducible pressure and modifier gradients and thus retention times were between 0.3 - 1.8% RSD. Alternatively, a needle pressure adjustment valve can also be used, which is driven by a solenoid and gave reproducible retention times of within 1% RSD (Saito et al. 1988).

1.5.2 Important Features of SFC

An important and often misinterpreted feature is that the supercritical behaviour is a new state and not a defined region in which the properties of liquids and gases become indistinguishable. Klesper and Schmitz (1987) had already stressed this point in 1987 and pointed out that diffusion coefficient and viscosity change gradually when pressure is above the critical pressure and the temperature is decreased from above the critical temperature to a temperature below the critical point (subcritical region). This is in contrast to a real state change such as vaporisation of a liquid in which the physical properties like diffusivity change dramatically and abruptly. Berger (1995) emphasised that the supercritical "state" is a defined state and that no phase transition takes place when going from supercritical to subcritical as one might assume when analysing phase diagrams in which the supercritical "state" is separated by phase borders. Considering a point at a temperature above T_c and a pressure far below P_c in the phase diagram in Figure 1.1, this marks the conditions of GC. If the pressure is isothermally increased, GC separation is turned into a SFC separation and by lowering the temperature the region of HPLC is reached. These changes happen without any phase transitions, but with gradually

changing physical properties of the mobile phases. This principle is the basis for unified chromatography, for which Martire and Boehm (1987) described the theoretical equilibrium between a mobile and stationary phase based on a lattice-fluid model. Tong et al. (1995) reviewed principles and applications of unified chromatography in an extensive review.

The reason why the supercritical "state" was misinterpreted was due to the dramatic increase in solubility in the critical region, which compared to the dramatic change in density when a phase transition from gas to liquid took place. However, this change takes place abruptly by crossing the phase boundary, whereas the change to the supercritical "state" is gradual.

Supercritical fluids offer intermediate viscosities and solute diffusivities between those of liquids and gases (Table 1.2), since efficiency is dependent on diffusivity and this in turn is dependent on viscosity, both of these will influence the efficiency of separation. Efficiency in packed columns can be described with the Knox equation (Berger 1995), since no analytical h_r-v equation is available. The Knox equation does not incorporate the variation of h with k which, however, is often not negligible in practice.

$$h_r = Av^{\frac{1}{3}} + \frac{B}{v} + Cv \qquad \text{eq. (1.12)}$$

with v the reduced linear velocity of the mobile phase and h_r the reduced plate height, given by the following equations

$$v = \frac{u\,d_p}{D_M} \qquad h_r = \frac{h}{d_p} \qquad \text{eq. (1.13)}$$

with u the linear velocity, d_p the particle size of the packing material, D_M the diffusion coefficient of the solute in the SCF and h the plate height. A in equation 1.12 represents the flow uniformity in the packed column, B the longitudinal diffusion and C the resistance to mass transfer in the mobile phase. Packed column SFC with its lower viscosity and diffusivity than HPLC has the same plate height at higher linear velocities (5-10 times higher), achieving the same separation 5-10 times faster (Schoenmakers 1993). The plate height can be expressed in the more common term of efficiency (N) by N = L/h with L the column length. The smaller the value of h the more efficient is a given column.

Efficiency can be defined in terms of peak broadening, which is expressed by sigma, σ, the standard deviation, which is a measure of peak width.

$$N = \frac{t_r}{\sigma} \qquad \text{eq. (1.14)}$$

with t_r, the retention time of a compound. Klesper (1978) noted that at high density the Reynolds number is above 10 and therefore turbulent flow was present in the packed column bed, increasing radial mass transport and relaxing flow profiles.

The effect of increasing density upon efficiency must be distinguished into two separate cases (Vérillon et al. 1992, Engelhardt et al. 1989). When a linear restrictor is used to maintain system pressure, an increase in density is achieved by an increase in flow rate, hence D_M decreases and u increases, this causes the last term (resistance of mass transfer to mobile phase) to increase, hence a lower linear velocity should be applied at higher densities (Shah and Taylor 1990). On the other hand, if backpressure regulators were used, a density increase was achieved by setting the backpressure regulator to a higher pressure, which decreased D_M. However, since the flow rate was constant, the linear velocity was reduced and this caused the h-u curve to rise only gradually.

An increase in temperature increased D_M and increased the linear velocity at the same time, hence increasing the efficiency (Engelhardt et al. 1989).

Low viscosities allow the linear velocity and thus flow rates to be increased to take advantage of high diffusivities whereas the pressure drop remains low compared to HPLC. High diffusivities and low viscosities, thus relatively low pressure drops allow the use of very long, efficient columns (Berger and Wilson 1993a). Nevertheless, there was controversy over the effect of pressure drop on efficiency. Schoenmakers and Unk (1987), Bartle et al. (1985) and Janssen et al. (1991a) investigated the effect of pressure drop on capacity factors and observed a detrimental effect on column efficiency. The latest knowledge of the pressure drop on column efficiency is that at high pressures the pressure drop across a packed column does not influence the column performance (Lee and Markides 1990, Pacholec et al. 1988, Engelhardt 1989, Gere et al. 1982). Nevertheless, the pressure drop determines the maximum number of plates possible in a column since it depends both on the pressure drop per theoretical plate and on the maximum allowed pressure drop (Schoenmakers et al. 1988).

Despite these advantages and the relative ease of retention control by adjustment of physical parameters and composition of mobile phase, only a few applications are used routinely (Villermet et al. 1991).

1.5.3 Packed Columns

In the early stages of SFC development only packed columns were used, however in 1981 with the introduction of capillary columns for SFC, interest in packed columns subsided and since then has experienced only a limited use (White 1987 cited in Pacholec et al. 1988). However, with the availability of instrumentation for packed column SFC the trend has reversed, and today 90% of the applications are performed on packed columns. Moreover, packed columns offer advantages over capillary columns, such as wider choice of

stationary phases, higher efficiency per unit time and higher loadability (Greibrokk et al. 1989, Engelhardt et al. 1989, Pacholec et al. 1988, Vérillon et al. 1992, Petersen 1990).

Packed column SFC is performed on columns with column packings and column dimensions optimised for HPLC. Totally porous silica and alumina are used in adsorption chromatography for non-polar and moderately polar compounds. The elutropic strength of supercritical fluid CO_2 at 50°C and 5000psi is only slightly higher than liquid pentane, and its solvent strength decreases rapidly below 1500psi (Berger and Deye 1990). This relatively low solvent strength prevents the elution of acidic, basic or strong hydrogen bonding compounds from silica columns as solutes with polar functional groups strongly interacting with silanol groups and therefore chemically bonded phases have been used for polar compounds .

Chemically bonded phases: Commercially available HPLC stationary phases were used, however the residual silanol groups present in these CBPs have a detrimental effect on peak shape and retention of polar compounds in SFC. This constitutes a serious drawback of packed columns compared to capillary columns, where fewer silanols are present due to considerably lower surface area (Schoenmakers et al. 1988). Because of steric hindrance it is impossible to derivatise all silanol groups on the silica surface using mono- or polyfunctional silane reagents (Nawrocki and Buszewski 1988). Since polyfunctional derivatising reagents introduce more silanols than the initial starting silanol due to hydrolysis of the unreacted chlorsilane groups, endcapping is obligatory (Unger and Roumeliotis 1976).

Using octadecyldimethylsilyl ligands only about half of the silanol groups are reacted, leaving a large number of residual silanol groups which are able to interact with polar analytes. So far only the aminopropyl group appears to shield the residual silanols from interacting with the solutes, and this has also been observed in normal phase chromatography (Greibrokk et al. 1989). The

pore dimension is a crucial parameter to be considered in achieving maximum possible coverage, as a small pore size (2nm) of silica hinders the octadecyldimethylsilyl reagent from diffusing into the pores, thus leaving a large number of residual silanol groups.

Different approaches were applied to circumvent the drawbacks of residual silanols, namely a) endcapping, b) production of polymeric phases, c) use of alternative phases based on non-silica material and d) addition of modifiers to mask residual silanols. Initial attempts involved the additional derivatisation of residual silanols, present after the initial derivatisation of silanols to produce the desired chemical bonded phase, with a smaller reagent such as trimethyl-chlorsilane. This procedure is called endcapping. Blilie and Greibrokk (1985) found that improved elution behaviour for PAH using an end-capped phase than for a conventional reverse-phase column. It was, however, noted that endcapping was unnecessary for bonded phases at maximum coverage on medium to wide pore silicas as no further derivatisation was observed (Poole et al. 1992).

Figge et al. (1986) and Engelhardt (1989) developed a new method to reduce residual silanols by producing GC-like polymeric stationary phases. For this, silica was mechanically coated with polysiloxane or butadiene prepolymer and then chemically crosslinked by the addition of peroxides or azo-t-butane. The underlying substrate was treated with silanes before the coating with the polymeric film in order to optimise the coverage. This provided a uniform coverage of the silanol groups and the treatment could be applied to substrates other than silica to obtain better pH stability. The possibility that the solute interacts with the substrate still exists, as the polymer film is permeable and only 1nm thick (Poole et al. 1992).

Payne et al. (1990) further confirmed that these polymeric phases produced by dehydrocondensation reaction yielded the most inert surface. This improved behaviour of the stationary phase and a considerable reduction in undesirable

solute/substrate interactions has also been confirmed by Taylor and Chang (1990). They further investigated the stability of the polymer columns and found no deterioration in efficiency when the columns were heated to 150 °C for 12 hours. The efficiencies of these columns were 2 -3 times higher than those of the conventional HPLC columns.

Another approach involves the use of columns which are based on different starting materials.

Porous graphitic columns: This packing material possesses a unique sponge-like structure with high mechanical stability. This has been produced by impregnating a silica template with phenol/hexamine mixture, polymerising this mixture within the pores of the silica gel and pyrolising the resin under nitrogen. The underlying silica template was dissolved and the packing material was finally heated to a temperature of 2000°C which left the porous carbon (Knox et al. 1986). In RP-HPLC, this material retained solutes far stronger than chemically bonded silica packings and polystyrene-divinyl benzene columns as shown by Bassler and Hartwick (1989).

Polymeric Packings: Polymeric materials have been developed for reverse-phase, ion-exchange, and size exclusion liquid chromatography. They are mainly based on polystyrene-divinylbenzene PS-DVB with various degrees of crosslinking. No silanol groups are present in these packing materials hence apolar partitioning, and according to the solute structure, π-π interactions are responsible for the retention (Villermet et al. 1991). One major disadvantage of these polymeric packings was their changing permeability when different modifiers were used and that the repeated expansion and compression during density programming can cause mechanical breakdown (Poole et al. 1992). Nevertheless, peak tailing and strong adsorption was also experienced with polymeric phases, which might be caused by metal impurities and/or polar compounds remaining from the polymerisation process or from oxidation of the sorbent surface (Henry cited in Pacholec 1988).

1.5.4 Retention Behaviour using pure CO_2

As in GC and HPLC gradients are also employable in SFC, however there is a greater choice of gradients which can be used in SFC, such as temperature, pressure/density, velocity and eluent composition. Klesper and Schmitz (1987, 1992) reviewed the possible gradients and also discussed the possibility of multiple simultaneous gradients. In order to employ gradients, the effect of each individual physical parameter should be known.

Density: As seen in Chapter 1.2.2, the solvent power is associated with the density and since retention is influenced by the mobile phase strength it is possible to control retention by changing density. Density can be increased by an increase in pressure and a decrease in temperature. Figure 1.2 shows that density increases rapidly with only very small changes of pressure at constant temperature around the critical point. If compounds are soluble in SC-CO_2 then there is a sharp increase in solubility around this point (Figure 1.7). It is however more advantageous to work in the region in which pressure causes a less dramatic increase in density allowing more control over retention.

An increase in density at constant temperature causes the solubility of a compound to increase in SCF and a concomitant decrease in the retention of the compound is observed when the compound is soluble, that is when its threshold pressure is exceeded (Giddings et al. 1969, Randall 1982, Peaden and Lee 1982). The effect of density on retention is mainly expressed as being almost proportional to the logarithm of capacity factor (k), hence nearly perfect linear plots are obtained when plotting log k versus density. This is however only correct over a limited region, whereas it is more accurate to use a quadratic fit when a wide density range is investigated (van Wasen et al. 1980, van Wasen and Schneider 1975).

Van Wasen further found that in plotting the logarithm of capacity factor (log k) versus the logarithm of density (log ρ) the isotherms of different compounds

converge at a higher density, thus the selectivity (relative retention of two compounds) is decreased at higher pressures. To achieve the highest selectivity it is best to conduct the separation at the lowest possible density (Lee and Markides 1994).

Since pressure and density are correlated to each other in a non-linear manner, a plot of log k versus pressure yields a non-linear plot (Mourier et al. 1986). However, as density is controlled by pressure one observes the same trends for increasing pressure as for increasing density, although to a different extent.

Temperature: The influence of the temperature is far more complex than the influence of density and pressure on retention. Two cases have to be distinguished: change of temperature at constant pressure or constant density. The case of changing temperature at constant pressure is the more complex as density changes at the same time.

Considering a fluid above the critical pressure and below or slightly above the critical temperature, a increase in temperature causes the density to decrease and hence retention to increase (Leyendecker 1993). At higher temperatures the vapour pressure of the solute becomes more pronounced, increases the solubility of the compound in the mobile phase and counteracts the loss in solubility due to density decrease. Moreover, the adsorption or absorption on or in the stationary phase is reduced at higher temperatures (Klepser and Schmitz 1987).

At low pressures the decrease in solubility caused by density decrease is dominant and hence an increase in retention with increasing temperature is observed . At moderate to high pressures on the other hand, density does not change so significantly with temperature (Figure 1.2), thus the capacity factor decreases at higher temperatures since the vapour pressure effect dominates (Novotny et al. 1971, Leyendecker et al. 1984). This results in a maximum of the

capacity factor with temperature at intermediate values. This behaviour has already been observed by Sie et al. (1966) and Figure 1.9 shows an example of the k dependency with temperature.

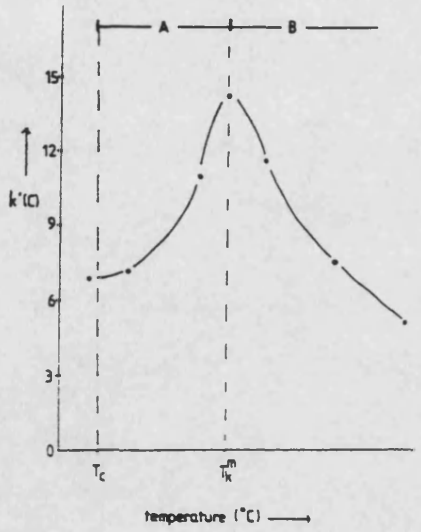


Figure 1.9 Dependency of retention factor on column temperature .

Starting at the temperature at which k is at its maxima and applying a negative temperature gradient results in a decrease in selectivity and resolution. However, when a positive gradient is applied from this point, the selectivity and resolution may increase due to a concomitant increase in diffusivity and viscosity (Klesper and Schmitz 1987).

Yonker et al. (1985), Bartle et al. (1988) and Chester and Innis (1985) explained the retention behaviour of compounds with temperature at constant pressure in terms of thermodynamic properties.

The approach of Chester and Innis expressed the temperature dependence in terms of the standard free energy, ΔG_T^0, for the partitioning process

$$\Delta G_T^0 = \Delta H_S^0 - \Delta H_m^0 \qquad \text{eq. (1.15)}$$

with ΔH_S^0 the standard enthalpy change in the stationary phase and ΔH_m^0 the enthalpy change in the mobile phase, entropy changes were neglected. Using the equation

$$\Delta G_T^0 = - RT \ln K = - RT \ln(k\,\beta) \qquad \text{eq. (1.16)}$$

with $K = c_s/c_m$ (c = concentration of the solute in the stationary and mobile phase respectively) and $\beta = V_m/V_s$ (V = volume of mobile and stationary phase respectively). Combining the two equations the following expression is obtained

$$\ln k = - \frac{\Delta H_S^0}{RT} + \frac{\Delta H_m^0}{RT} - \ln\beta \qquad \text{eq. (1.17)}$$

The first and last term on the right hand side represent the capacity factor under GC conditions. In SCF, β and ΔH_S^0 can change with temperature due to adsorption of mobile phase onto the stationary phase. Moreover, ΔH_m^0 is dependent on density and since density changes the enthalpy changes accordingly. This equation is therefore more appropriate in describing the dependency of k with temperature at constant density.

When temperature is changed at a constant density then the behaviour is less complex, since the solvent strength due to density remains constant. An increase in temperature enhances the vapour pressure and reduces the adsorption or absorption on or in the stationary phase, resulting in a decrease of k' (Klesper and Schmitz 1987).

Klesper and Schmitz (1987) derived an equation for the change in selectivities with increasing temperature. They concluded that selectivity change is

influenced by the extent to which the retention times of the two compounds are determined by the volatility increase and solvation decrease with increasing temperature.

Yonker and Smith (1986) expressed retention in terms of entropies and enthalpies of transfer from the mobile phase to the stationary phase

$$\ln k' = - \frac{\Delta H_T^0}{RT} + \frac{\Delta S_T^0}{R} - \ln\beta \qquad \text{eq. (1.18)}$$

with ΔH_T^0 the enthalpy of transfer and S_T^0 the entropy of transfer. When plotting ln k' versus 1/T a so called van 't Hoff plot is obtained and the gradient yields ΔH_T^0 according to Yonker and Smith (1986). Roth (1991, 1993) pointed out that gradients of van' t Hoff plots do not yield ΔH_T^0 alone but also contain volume change of transfer. Nevertheless, many scientists have used the above equation to obtain ΔH_T^0.

Retention mechanisms: Sakaki et al. (1994) used the equation of Roth and showed that the retention behaviour of nonpolar compounds on ODS-columns using CO_2 and N_2O can be explained by different partitioning in the stationary phase and solubility in the mobile phase. There was no difference in the interaction of the two mobile phases (CO_2 + N_2O) with the stationary phase. However, when polar columns were used, the mobile phases seemed to interact differently with the stationary phase, thus influencing the retention differently. Sakai's group explained the results in terms of normal phase chromatography.

Schoenmakers et al. (1988) explained the retention behaviour as a combination of adsorption mechanisms, where one is caused by residual silanols and the other due to interaction with the chemically bonded stationary phase. They calculated a parameter which served as an indication of the number of accessible silanol groups for different columns by investigation the effect of sample size on

retention. Shang et al. (1994) used van 't Hoff plots to evaluate stationary phases regarding residual silanols. Strong interactions between polar solutes and residual silanols were observed at low densities, causing a dual retention mechanism. Van 't Hoff plots showed extrapolated intersection points which could be related to hydroxyl surface concentration and allowed a packing material to be characterised.

Efficiency: Berger (1993) investigated the effect of adsorbed mobile phase components on stationary phase upon efficiency and explained the cause for efficiency loss at low temperature and pressure. Strubinger et al. (1991a) measured distribution isotherms of pure CO_2 on several stationary phases, such as liquid stationary phases in capillary columns and silica and ODS stationary phases in packed columns. They demonstrated excessive adsorption at low temperature and pressure on all stationary phases, which decrease with increasing temperature and increasing density. Maximum adsorption was at about 0.35g/ml on ODS and silica material. At about the same density Berger (1993) observed a break in the linearity of log k versus density, which were linear below and above this density. He argued that the increasing layer of adsorbed mobile phase caused the non-linearity often observed in log k versus density plots and the loss in efficiency at these conditions due to the unfavourable diffusion in the thick adsorbed CO_2 layer.

1.5.5 Retention using Modified Fluid Mixtures

As discussed in chapter 1.4.3 it is impossible to derivatise all residual silanols present in chemically bonded phases, causing strong interactions with polar compounds, therefore the addition of modifier is required for the elution of polar compounds (Greibrokk 1993). Berger and Deye (1990) summarised the effects of modifier additions in supercritical fluid chromatography; a) coverage of active sites, b) swelling or modifying the stationary phase, c) increasing the

mobile phase density and d) increasing the solvent strength of the mobile phase. Moreover, the addition of a modifier will also affect the critical parameters of the fluid mixture. In summation, the addition of modifiers causes changes in the mobile and stationary phase (Janssen et al. 1989, Levy and Ritchey 1986).

The influence of temperature, pressure and density is comparable to pure CO_2, however it has been noted that the influence of pressure and density is smaller in mixed phases as the pressures applied are high and therefore the density no longer changes considerably with pressure (Berger and Deye 1990).

Modification of mobile phase: In order to interpret the influence of the modifier on retention solvatochromic dyes are used to measure interactions between modified fluids and a dye and correlate the transition energies to capacity factors. Deye et al. (1990) used Nile Red for their measurements as they noted that the polarity parameter P' which is based on experimental solubility data by Rohrschneider, did not reflect the dramatic change in solvent strength experienced when adding MeOH to CO_2. Furthermore, even though large changes in retention were observed in adsorption chromatography, the frequently applied equations incorporate only the adsorption of polar modifier onto the surface, however neglected the mobile phase interactions with the solute. Deye et al. (1990) emphasised that by using solvatochromic dyes solvent strength only is measured and not elution strength as in adsorption chromatography. Clustering around the polar solute was incorporated in the measurements and the following trend was observed: the greater the difference in polarity between the components, the more intense the clustering and thus the greater the non-linearity between solvent strength and composition. The measurement also showed that changes in temperature and pressure had only small effects on retention. Although density determined the nature of the modifier cluster, it only had a secondary effect on retention. When log k was plotted versus transition energies $E_{(NR)}$ residual non-linearities were observed, suggesting that modifier-stationary phase interactions were not included. The authors concluded that if packed columns followed a bonded phase

chromatography (BPC) mechanism, the equation has to account for the difference of local and bulk composition and if adsorption mechanisms were dominant, then the equations have to be extended to incorporate solute-solvent interactions in the mobile phase. The most likely mechanism may be a mixture of the two.

Changes in viscosities and diffusivities with mixed mobile phases have not been explicitly investigated, but increasing the modifier concentration in the mobile phase will increase the viscosity. This will increase the pressure drop over the column according to Darcy's law, however no loss in efficiency was observed (Köhler et al. 1994). Moreover, Berger (1991) did not note any significant change in efficiency when the modifier was increased causing the separation at higher concentrations to be performed at subcritical conditions. If separations were however conducted in the region of the vapour-liquid equilibrium zone phase, separation would take place causing noisy baseline and loss in efficiency (Berger 1991, Page et al. 1992).

Modifications of stationary phase: Chemically bonded phases contain heterogeneous surfaces due to steric reasons which does not allow a complete derivatisation of all silanols present. As pointed out by Schoenmakers et al. (1988) both the chemically bonded phase and the residual silanols contributed to retention, resulting in poor peak shapes if two mechanisms with different adsorption isotherms are present. Thus, the interaction of sample proton donor/acceptor and dipolar groups with residual silanol groups causes peak tailing and sample adsorption (Dean and Poole 1989, Nomura et al. 1989, Ashraf-Khorassani and Taylor 1988a). The role of the modifier was therefore considered as deactivating the residual silanols. Janssen et al. (1989, 1991b) measured adsorption isotherms for various modifiers and concluded that the large changes in retention times by adding small concentrations of modifier was caused by the deactivation of active sites. They further attempted to establish the relative contribution of modifier to mobile and stationary phase and concluded that the stationary phase modification was the prevailing effect of polar modifiers, as

more polar compounds can be eluted from capillary columns since fewer silanol groups were present (Janssen et al. 1991b). Furthermore, they observed that the modifier influenced retention more at lower temperatures since the contribution of adsorption to retention was greatest at low temperature. Blilie and Greibrokk (1985) and Upnmoor and Brunner (1989) concluded that the effect of modifiers were primarily due to interaction of the modifier with the stationary phase, which was confirmed by Geiser et al. (1988) when using water as the stationary phase modifier to deactivate active silanols.

Berger and Deye (1990), however demonstrated that the difference between packed and stationary phases were due to the large changes in phase ratio and not due to the amount of silanol groups. Silanol interactions only played a dominant role at modifier concentrations below 1 - 2% due to the fact that no monolayer of modifier was absorbed on to the stationary phase and for stationary phases being less polar than the solutes (Berger and Deye 1991a). For more polar stationary phases the interactions with silanols became less significant than in nonpolar phases, since higher amounts of modifiers were adsorbed on the more polar stationary phases (Strubinger et al. 1991b, Lochmüller and Mink 1989). The adsorbed mobile phase components were considered to be the primary stationary phase since very different columns produced surprisingly similar selectivity and the separation mechanism involved a single fluid-fluid partition mechanism (Berger and Deye 1992). Berger and Deye (1991a) used different approaches to separate polar solutes on various columns and concluded that the separation of polar solutes required the use of polar columns in conjunction with polar modifiers in order to obtain good resolution and peak shapes. For very polar solutes which are ionised during separation the addition of small amounts of a very polar additive to the modifier was required in order to obtain satisfactory peak shape and resolution (Berger 1995). The role of the additives were considered to be multiple, namely; a) coverage of active sites, b) changing the polarity of the stationary phase, c)

suppression of solute ionisation and d) increasing the solvent strength of the mobile phase.

The addition of modifiers or additives to the mobile phase sometimes produced permanent changes in the stationary phase. Schmitz et al. (1987) noted the permanent modification of a silica stationary phase upon exposure to 1,4-dioxane at elevated temperatures. Greibrokk (1993) and Smith and Briggs (1994) noted the damage to a silica column and a cyano column when methanol and citric acid respectively were used as additives.

Efficiency: Berger and Deye (1991b) and Ashraf-Khorassani et al. (1990) did not observe any efficiency loss when modifiers were used even at relatively high concentrations. Efficiency losses were only experienced when the modified fluid separated into two phases, the solubility of a solute in the mobile phase was insufficient (Berger and Deye 1991b), or when the stationary phase polarity did not match the analyte polarity (Ashraf-Khorassani et al. 1990).

Retention mechanisms: Villermet et al. (1991) investigated the retention behaviour of substituted PAHs on silica and a polystyrene-divinylbenzene column using a mixed mobile phase. They concluded that the reduction in retention times was caused by the increased density of a mixed mobile phase compared to a pure CO_2 mobile phase because the retention reduction with increase in density was of the same order of magnitude on silica with or without a modifier in the mobile phase. Berger and Deye (1990) however demonstrated that density has a far smaller influence on retention compared to composition. They investigated the retention at constant density with increasing concentrations of modifier and at constant composition with increasing density. This indicated clearly the stronger influence of composition on retention than density. Villermet et al. (1991) noted that the vapour pressure of the solutes played a significant role on the retention behaviour. Maxima in capacity factors could be observed on silica with increasing temperature and the higher the boiling point, the higher the temperature at which the maxima occurred. In

contrast, the capacity factors on the polystyrene-divinylbenzene columns showed no maxima, therefore the interactions with the stationary phase had a stronger influence on the partition mechanism.

Smith and Sanagi (1990) derived from experiments that the retention of the alkyl aryl ketones on a ODS column were determined by volatility and a normal-phase polar-polar interaction between the analytes and active sites on the stationary phase. With higher homologues the retention increased with increasing carbon number and volatility was thereby the dominant parameter in retention. However, at higher methanol concentrations the influence of chain length decreased. This theory was further investigated for the separation of homologous aromatic alcohols and carboxylic acids on a ODS, a PS-DVB, a cyano and an amino-propyl column using a modified mobile phase (Smith and Briggs 1994). Using the ODS column they found little difference between the homologous phenylalcohols when a modifier was added, suggesting little influence of volatility on retention. In the case of the phenylalkanoic acids, a decrease with increasing carbon number was noted, suggesting that polarity effects were dominant and that the addition of methanol deactivated free silanols. However, a good correlation between log k and carbon number was observed for aryl alkyl ketones, phenylalkanols and phenylalkanoic acids on the PS-DVB column. The addition of methanol was thought to alter solubilising properties rather than deactivating strong surface interactions, and it appeared that the elution of the homologues was primarily determined by their volatility. Nevertheless, a temperature increase which should increase the volatility resulted in a decrease in retention, suggesting the decrease in solubility was more important than the increase in analyte volatility. A dependence on the carbon chain length was also observed on the aminopropyl column for the phenylalkanols, whereas the retention on the cyano column must be influenced by polar interactions, as the alkanols eluted significantly later than the ketones despite having comparable boiling points.

Mourier et al. (1986) differentiated the retention mechanism on silica and ODS columns, by applying Snyder's theory to silica columns and a nonpolar-nonpolar retention mechanism to ODS columns. They emphasised the importance of the type of modifier on silica columns since it was able to influence selectivity dramatically which depended on the modifiers' ability to deactivate residual silanols. Moreover, if solutes were substantially more polar than either of the two phase, interaction with active sites tended to be the primary interaction site and deactivation became progressively more important (Lee and Markides 1986, Smith et al. 1985). Berger and Deye (1992) recognise that silanophilic interactions were important, however stressed that in order to investigate retention mechanisms in modified phases, these interactions must be suppressed, so that one retention mechanism prevailed and allowed the investigation of this mechanism. They found a dependence of retention on the surface area of diol columns and obtained good correlation for a variety of compounds. They concluded that changing the pore diameter indicated another method of changing retention and recommended the use of smaller pore diameters since highly efficient separations were observed on these columns. Gere et al. (1982) achieved higher efficiency with columns using small particle diameters.

Heaton et al. (1994a) based their retention model on molecular interactions of the solute with transfer energies of adsorption and solution. These energies were related to solubility parameters, which were either calculated or measured, and related to a capacity factor by the equation of Karger and Snyder (1978). Coefficients were deduced for each temperature and mobile/stationary phase and in turn these values were used to predict retention behaviour. Reasonable agreement between predicted and measured capacity factors were found for monosubstituted benzenes on an ODS-column, however, with disubstituted benzenes it was not possible to predict the elution order.

1.5.6 Detectors

One of the main attractions of SFC is the applicability of GC and HPLC detectors. Additional benefits are that thermolabile or high molecular weight compounds can be separated at lower temperatures than in GC, but the same detectors can be utilised, making SFC a very versatile separation technique with the possibility of multiple detection (Munder 1991). Detectors can be differentiated into optical and ionisation based detectors, in which the optical detection, except in the case of the light scattering and ion mobility detection, are performed in closed cells. The most common optical detectors include UV-, fluorescence, light scattering and chemiluminescence detection (Bornhop and Wangsgaard 1989, Lee and Markides 1990). Depressurisation takes place after the detection, therefore the detection is performed in solution and variable restrictors can be used. On the other hand, open cell detection is mainly applied for GC based detectors where depressurisation takes place just before the detector cell.

UV-detection: UV-detectors together with FID detectors were one of the first detection methods applied in SFC (Wilsch and Schneider 1986), and since then have found wide acceptance. UV-detection is the detector of choice for packed columns since it is able to cope with high flow rates when using 4.6 mm i. d. columns and this also allows the use of a detector cell volume of 10µl without causing excessive band-broadening. When using capillary columns, the detector cell volume has to be minimised and a detector volume of 50nl to 270nl was recommended by Peaden and Lee (1983) in order to produce not more than a 1% resolution loss. UV- detection however has the drawback of being a specific detector, thus UV absorbing chromophores are necessary for a compound to be detected. This can nevertheless be an advantage as various modifiers can be added to the primary fluid without an increase in baseline noise. A negative baseline shift was observed when density programming was performed using pure CO_2 due to refractive index changes (Fields et al. 1988). Baseline drifts could also be observed when using modified fluid mixtures, however the extent

and direction depended on the modifier used. Giorgetti et al. (1989) obtained a negative baseline drift when methanol was used in a pressure programmed run and a positive baseline rise with 2-propanol. The problem could be alleviated by thermostating the UV cell with cool H_2O, thus reducing refractive index effects (Fields et al. 1988).

Fluorescence: The fluorescence detector, as already used in HPLC offers a very sensitive and selective method for the analysis of native fluorescent analytes or those which can be derivatised with a fluorescent reagent. Although only a few applications using fluorescence detectors have been published in SFC (Bornhop and Wangsgaard 1989) there is great potential in the use of this detector. A variety of polar compounds have been found to be difficult to separate using SFC, therefore derivatisation of these compounds could be used both to lower their polarity and to produce a fluorescent product.

Chemilumiscence: In a chemiluminescence detector molecules are transferred to an excited state by a chemical reaction and light is emitted while returning to the ground state. Low pressures in the detector is needed to reduce secondary reactions in which the excited molecules collide with each other or the detector walls. The detector is mainly used as a selective detector measuring sulfur chemiluminescence which is based on the reaction of sulphur monoxide with ozone or on the reaction of a sulphur containing compound with fluorine. Sulphur monoxide is produced by combustion of sulfur containing compounds in a hydrogen-oxygen flame. Chang and Taylor (1990) used this configuration to detect very low levels (35pg) of sulphur. After flame optimisation it was possible to detect minimum amounts of 10-1400pg of aromatic sulphur compounds (Pekay and Olesik 1990). However, the use of chemiluminescence detectors is mainly restricted to capillary columns as the signal is reduced substantially by the high CO_2 flow rates due to quenching (Foreman et al. 1988). Advances made by Howard and Taylor (1993) demonstrated that the greater flow present with packed column SFC and the addition of a modifier did not quench the signal to a greater extent than in capillary SFC.

Light scattering detection: The three detectors mentioned above are selective detectors, the light scatter detector however enables universal detection and is therefore particularly promising for use in SFC. The light scattering detector is based on the nebulising of the column effluent, evaporating the solvent from the droplets generated in the nebulizer, and measuring the scattered incident light beam. In SFC it is not necessary to add nebulising gas since the fluid expands to high enough flow rates (Lee and Markides 1990). The temperature in the heated drift tube, in which the evaporation of the solvent takes place, must be controlled carefully in order to avoid sample loss of volatile compounds due to evaporation, however it has to be high enough to circumvent the formation of solid CO_2 particles (Cocks and Smith 1991). Carraud et al. (1987) were the first to achieve a coupling of SFC on packed columns using CO_2 with LSD. They found a non-linear LSD response, however quantification was possible when using a logarithmic scale for the concentration axis as a straight line was obtained. One of the major drawbacks was that the response of the LSD was dependent on the CO_2 velocity and thus pressure programming was precluded, when quantitative results were required. Contrarily, Hoffmann and Greibrokk (1989) produced reproducible results in both peak area and retention times even when pressure gradients using a modified mobile phase was applied. LSD detection was further applied to the analysis of sugars, however there was no reference made towards quantification (Herbreteau et al. 1990). Cocks and Smith (1991) used an LSD detector for the detection of fatty acid methyl esters and pointed out that the amount of light scattering was dependent on the size of the nebulised particles and to a certain extent on the wavelength of the incident light and the detection angle.

Flame ionisation detection: In the early development of SFC, the FID was mainly used in conjunction with conventionally packed HPLC columns, which caused severe problems due to the high flow rates (Giddings et al. 1968). The initial problem of detector spiking however was caused by the use of long linear restrictors, providing a long decompression zone which caused precipitation and

clustering of the analyte in the restrictor. This has since been overcome by improved restrictor technology, which has been developed by several researchers (Berger 1989b, Chester et al 1985, Guthrie and Schwartz 1986). The main interest presently is in the use of packed capillary columns (0.25mm i. d. packed with 5-10μm) because of their better sample loadabilities than capillaries and their lower flow rates, higher permeabilities, and hence greater efficiency than conventionally packed columns (Hirata et al. 1988). Hirata et al. (1988) and Schwartz (1988) were successful in coupling microbore columns directly to an unmodified FID. Richter (1989) improved the FID detector design for packed columns by adding an extra heating source for heating the restrictor to circumvent the effect of cold spots. Cold spots in the detector causes high molecular weight compounds to precipitate, causing splitting of peak. The main drawbacks of the FID are that it firstly cannot be used when modifiers are added to the mobile phase, because of the increased background noise and hence decreased overall sensitivity. The FID, however, allows the use of water and formic acid as modifiers and the former has been successfully applied in the separation of fatty acids (Geiser et al. 1988). Secondly, being a universal detector, the FID requires the use of highly pure carbon dioxide as any contamination of organic origin increases the baseline noise and therefore reduces the overall performance.

Thermionic ionisation detector: the TID is often referred to as a nitrogen-phosphorous detector (NPD) due to its sensitivity towards nitrogen and phosphor. This detector is the detector of choice for sensitive and selective trace analysis for pesticides containing nitrogen and phosphorous. Fjeldsted et al. (1983) was the first to employ TID for SFC and observed good selectivity and performance without any problems when the NPD was used in SFC. The NPD is based on a similar principle as the FID, however with the exception that an alkali salt bead is present above the flame jet in the combustion chamber. Mostly a rubidium silicate bead is electronically heated to 600-800°C and a plasma is sustained in this region by the supply of hydrogen and air gas flows.

Alkali metal ions emitted from the bead ionise nitrogen or phosphor containing compounds and these are subsequently detected. There are several modifications of the TID, e. g. Kolb-Bischof and Patterson types (Dressler 1986). West and Lee (1986) used a Patterson type NPD for the detection of nitrated polycyclic aromatic compounds and also mentioned the use of methanol as modifier. They used three modification of the detector and noted that the best selectivity and stable baseline during pressure programming was obtained using the nitro-selective mode of detection. The conventional mode of the NPD using hydrogen and air was very sensitive to mobile phase density changes and hence its application was very restricted. David and Novotny (1988) used a Kolb-Bischof type detector for the derivatised α-keto acids and optimised gas flow rates, thermionic source composition and mobile phase composition in a later publication (David and Novotny 1989). They observed no significant increase in detector background noise when up to 10% modifier was added. Greibrokk et al. (1987) was the first to report the possibility of using modifiers of up to 7% methanol without any detrimental effects on the detector baseline noise. Mol et al. (1991) investigated the effect of modifier, bead position and flow rates of hydrogen and air on the response in a flame-based NPD using packed-capillary columns. They noted a decrease in the lowest detection limit when the modifier concentration was increased to about 6%. Berger et al. (1994) investigated the use of NPD combined with UV detection for the analysis of carbamate pesticides using packed columns. They observed detection limits of 100ppt, however noted that at MeOH concentrations greater than 20% the NPD bead tended to be deactivated, thus a maximum of 10% MeOH modifier was used.

Electron capture detector: The ECD is one of the most sensitive detectors in GC (Dressler 1986). The Ni^{63}-foil in the detector emits low energy β-particles to ionise the make-up gas, which produces secondary thermal electrons and results in a constant current. If electron-capturing compounds enter the ECD the thermal electrons are captured giving either negative molecular ions or negative fragments, which decreases the background current and can be registered.

The use of ECD in SFC was first suggested by Richter et al. (cited as personal communication in Later et al. (1987) using pure CO_2 and Kornfeld (1992 cited in Strode and Taylor 1991) was the first to use mixed mobile phases in conjunction with ECD. Chang and Taylor (1990) reported the influence of density of the mobile phase using pure CO_2 on the ECD response. They further explained the increasing baseline with pressure with previously reported properties of CO_2, such as being a weak electron-capturing gas and reducing the diffusion coefficient of thermal electrons. Strode et al. (1994) reported on the analysis of felodipine by packed column SFC using ECD and UV detection. They used up to 8% MeOH and found the separation using SFC a more robust method than the HPLC method. In a later publication they optimised modifier concentration, modifier type, detector temperature and make-up gas flow rate (Strode and Taylor 1996). Methanol was used as a modifier, causing increasing background noise with rising concentration due to the electron scavenging of MeOH. The optimisation of the detector temperature revealed the presence of two electron capture mechanisms, namely non-dissociative and dissociative modes.

Detailed reviews on detectors used in SFC and their potential use are published by Lee and Markides (1990), Richter et al. (1989) and Bornhop and Wangsgaard (1989). Di Maso et al. (1990) and Dressman and Michael (1995) reported the potential of electrochemical detection in SFC and Huang et al. (1991) about ion mobility.

1.7 Supercritical Fluid Extraction

The use of SFE for sample preparation increased substantially in the 1980's as SFE is less time consuming and more selective for the extraction of a variety of compounds. Sample preparation in analytical chemistry often requires several steps such as liquid extraction followed by filtration and sample clean-up, making this process more time consuming than the actual analysis and data

handling (King and France 1992). Conventional solvent extraction frequently involves the use of considerable quantities of solvents often chlorinated, which are a potential health hazard and require specialised disposal. The relatively new technique of SFE offers an alternative as CO_2 is primarily used as solvent due to its favourable properties. These are: a) a moderate critical pressure (73.8 bar), b) low critical temperature (31.1°C), allowing the extraction of thermally labile compounds, c) low toxicity and reactivity, allowing it to be vented into the atmosphere, d) high purity at low cost, e) good solubility of non polar and medium polar compounds and f) compounds are not exposed to light and oxygen during the extraction (Dean and Kane 1993). Moreover, lengthy extraction times associated with Soxhlet extraction, can be reduced by using SFE (Richards and Campbell 1991).

1.7.1 Instrumental Requirements for SFE

The basic requirements for the application of SFE are almost identical to that for SFC except that the column is replaced by a sample cell and a collection device is required for the extracted compounds. The cells are mostly made of stainless steel or alternative non-extractable material, with sizes from 150µl - 50ml (Dean and Kane 1993) and able to withstand high pressures. There has however been some controversy about the influence of cell geometry and cell orientation on recovery. Dean and Kane (1993) reported about various extractions, in which the cell geometry played a significant part in the resultant recovery. Hawthorne et al. (1993) however concluded that cell geometry and orientation did not influence extraction recovery when the cells were packed so that no excessive dead volume was present, and that the cost and convenience to fill the cell was more important. Hawthorne et al. further recommended that the extraction cell should be installed in such a way that the flow of the SCF flows vertically downwards through the cell, so that dead volume cannot have any effect on the recovery.

Two methodologies have been developed for the trapping of the extracted analyte after depressurisation. The solute is either transferred directly into a chromatographic system (on-line) or is collected off-line for subsequent analysis to be carried out. Since on-line trapping has not been applied in this thesis, no detailed discussion is given. However, there are numerous treatises about on-line coupling to a variety of chromatographic and spectroscopic instruments (Greibrokk 1995, Stuart et al. 1996, Vannoort et al. 1990 and Westwood 1993).

Off-line trapping can be subdivided into cryogenic, solvent and solid phase trapping. The methodology of cryogenic trapping was used by Richard and Campbell (1991) with the addition of a collection flask to improve the recoveries of the more volatile compounds at a temperature of -50 to -30°C and achieved recoveries of 80 - 100%. Cryogenic trapping involves the cooling of a vial or flask, in which the supercritical fluid is transferred while depressurisation takes place. The cooling is achieved either with liquid nitrogen or CO_2. Hawthorne (1993) recommended cryogenic trapping only for non-volatile samples, as the recovery for volatile samples using this technique was poor due to aerosol formation.

An alternative method is to place the outlet of the restrictor into solvent trapping the analyte, while the decompressed fluid vents to the atmosphere. The maximum flow rate with this technique is about 1-2ml/min as 1ml of fluid expands to 500ml gas and therefore higher flow rates would result in violent bubbling, causing loss of analyte (Camel et al. 1993 and Pipkin 1992). However, solvent trapping is regarded as the simplest method of trapping solutes due to the relatively uncomplicated optimisation procedure. Porter et al (1992) conducted a thorough study of all the parameters which might influence recovery, such as effect of solvent depth, solvent temperature, mechanical stirring and presence of inert bodies in the trapping solvent. The solvent height had a significant influence on the recovery, however additional stirring did not improve the recovery. As the depressurisation step cools down the restrictor and solvent due to the Joule Thompson expansion effect, there is a need for restrictor

heating in order to avoid restrictor plugging. This however can cause loss of volatile compounds. Porter et al. (1992) developed a novel solvent trapping device, which allows the entire restrictor to be heated while the trapping solvent is being cooled. The colder temperature of the collection solvent improves the recovery of the analyte due an increase in viscosity, which causes the formation of smaller bubbles in the solvent, creating a greater surface : volume ratio and hence results in better contact with the solvent. Thompson et al. (1993) studied the effect of numerous solvents, however were unable to relate high recoveries with the viscosity of the individual solvents. They proposed the analyte should have a high solubility in the solvent to enhance the interaction. In addition, Langenfeld et al. (1992) could only find a small dependence of solvent height on recovery and found no correlation of recovery on cell geometry, when the extraction cell was filled completely so that no excessive dead volumes were present.

Solid phase trapping is considered a more complicated way of trapping solutes, as it requires a high degree of optimisation, such as choice of trapping material, SCF modifier, trapping temperature and elution solvent (Bowadt et al. 1989). Two mechanisms take place concomitantly during solid phase trapping. The expanding extraction fluid cools down the trap containing the solid phase material, thereby achieving cryogenic cooling while the solid assists the trapping of the analyte. Bowadt et al. (1989) optimised the trapping of PCBs using pure CO_2 and modified CO_2. The temperature of the trap was irrelevant when using pure CO_2, however when 2% MeOH was added, the temperature needed to be high enough to prevent condensation of the MeOH, as otherwise the condensed MeOH acted as an elution solvent. The choice of the elution solvent was crucial, as considerable amounts of analyte could remain adsorbed on the trap, if the elution solvent was too weak to compete with the solute - solid phase interactions

1.7.2 Parameters Influencing Recovery

In depth discussions about parameters influencing extraction can be found in numerous reviews of the use of analytical SFE for the extraction of environmental samples (Hawthorne et al. 1993, Camel et al. 1993, Janda et al. 1993a,b, Bowadt and Hawthorne et al. 1995).

Clifford (1993) proposed a SFE triangle as seen in Figure 1.10, which shows the interrelation of the factors influencing recovery. In the following section various parameters which influence extraction recovery will be discussed with reference to the three points in the triangle, namely solubility, matrix and diffusion. However, Hawthorne (1993) extended the triangle since the transport of the extracted compounds from the extraction cell to the outlet and the quantitative collection of the compounds also influenced the degree of recovery.

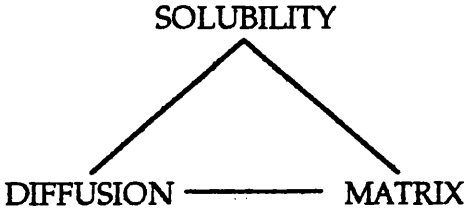


Figure 1.10 SFE triangle

Pressure or density: The influence of pressure and density upon solubility have been discussed in section 1.4. Before optimisation of the parameters in SFE can be undertaken, the objective of the extraction has to be considered. It has to be decided whether a selective extraction which pre-separates the analyte from the contaminants and therefore renders sample clean-up unnecessary or reduces it to a minimum would be desirable. Conversely, a fast extraction may be preferred, which involves the application of high pressures to achieve maximum solubility. This however, may also increase the amount of co-extractants. These may not interfere with the determination of the solute or may require only a short sample clean-up.

King (1989) identified three characteristic pressure ranges, namely threshold pressure, fractionation pressure and the pressure at which maximum solubility occurred. The threshold pressure is the pressure at which the extracted compound can be detected, however the determined pressure depends on the sensitivity of the detection method. In order to extract any compound the threshold pressure must be exceeded. The fractionation pressure encompasses the range from the threshold pressure to the pressure at which maximum solubility occurs. In this range the largest change in solubility takes place and therefore it may be possible to selectively extract compounds, however only under the precondition that there is sufficient difference in molecular weight and polarity (King and France 1992).

The belief that solubility is primarily dependant on density, only holds true for non polar to medium polar compounds, since an increase in density results in increased interaction and the solubility curve of non polar compounds mirror the density increase with pressure. Lee and Markides (1990) noted that CO_2 has a comparable density to liquids, however density does not reflect intermolecular attraction. For polar compounds the addition of a modifier has a far more profound effect, as an increase in pressure enhances solubility only to a limited extent.

Maximum solubility, desired in process application of SFE, is only important in the initial stage of the extraction (Clifford 1993), where a considerable amount of compound has to be extracted due the increased concentration of the compound on the matrix surface. As seen from Figure 1.10 solubility is only important for the extraction of the first 50% of the analyte, the extraction becomes diffusion controlled and solubility is no longer rate-determining. The percentage extracted until the extraction becomes diffusion controlled is dependant on the sample and distribution of the analyte in the matrix. Furthermore, extractions can be carried out below the maximum pressure, if the concentration of the analyte is relatively low and time is not the only criterion. Additionally extraction at very high pressures can have a negative effect on

extraction. Increasing pressure above the level necessary for maximum solubility, can cause the salting out of the solute from the dense fluids due to the dominance of repulsive forces (Lee and Markides 1990).

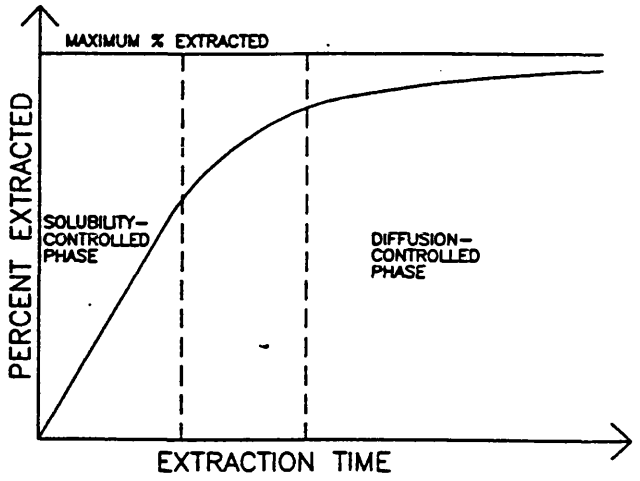


Figure 1.10 Extraction recovery

The calculation of these pressures was derived by King (1989) and solubility was measured using various techniques, however Lee and Markides (1990) noted appropriately

".....the rapid demand for results in many applied analytical chemistry situations leaves limited time to apply complex theoretical calculations to the problem at hand." (Lee and Markides 1990).

In addition to the complexity of these calculations and the need for appropriate computer capacity, the applicability of these results to practical problems is often questionable. An example of this was observed for the extraction of PAHs. Despite sufficient solubility of PAHs in CO_2 at the applied conditions, the recovery of PAHs from incinerator fly-ash was low, as CO_2 was unable to compete with the active sites on the matrix (Alexandrou and Pawliszyn 1989 cited in Hawthorne et al. 1993). It is increasingly recognised that high solubility of a compound in SCFs is not the sole parameter to achieve high recovery, but the ability of SCF to overcome matrix-analyte interactions is often

more important to achieve quantitative recovery (Hawthorne et al. 1993). This exemplifies the dominance of matrix effects in analytical SFE, limiting the applicability of theoretically calculated solubilities. Additionally, the calculated values of solubilities and experimental conditions do not take the presence of other extractable material into account, which can either enhance or decrease the solubility of the desired compound.

Flow rate: Generally, extraction can be divided into two categories, namely extractions in which large quantities have to be extracted or in samples with minor contamination. In the case of highly concentrated samples, a flow rate increase will increase the extraction as more solute can be solubilised at a unit time. Maximum solubility of the compounds in these type of extraction will ensure fast extraction rates. On the other hand, when small concentrations of solute are present, then these can readily interact with active sites on the matrix. An increase in flow rate does not accelerate the extraction since the extraction is not solubility limited but kinetically limited. In extraction of this nature, it is beneficial to apply a static extraction mode, in which the flow rate through the cell is stopped, allowing better penetration of the matrix with fluid and possible swelling of the matrix, so that diffusion out of the pores is enhanced.

Temperature: An increase in temperature increases vapour pressure and hence is able to enhance solubility, however this is counteracted by the drop in density. Moreover, temperature is able to overcome a weak energy barrier of desorption, however for stronger interactions the use of a specific modifier was recommended (Alexandrou et al. 1992). Langenfeld et al. (1993) studied the extraction efficiency of PCBs and PAHs and found that PCBs were efficiently extracted at temperature of 200°C, demonstrating that temperature can enhance the desorption process. Bowadt and Hawthorne (1995) outlined in their extensive review of SFE in environmental analysis the possibility of applying high temperatures as a more effective means of overcoming strong interactions

than an increase in pressure and may in certain applications be used as a possible alternative to modifiers.

Modifier: Langenfeld et al. (1994) investigated the influence of several modifiers on the extraction of environmental samples. The group outlined possible ways of modifier interactions which assisted the extraction, namely a dramatic increase in bulk solubility which influenced the matrix/fluid distribution and therefore caused a preferred partitioning of the analyte into the SCF. Furthermore, the modifier was capable of deactivating active matrix sites even at low concentrations, hence only small additions of modifiers were necessary when the extraction was limited by slow desorption kinetics. Accelerating the desorption process can also be achieved by the modifier through interaction with the analyte/matrix complex. Moreover, the addition of modifier to the matrix may swell the matrix, allowing the fluid to access remote pores and assist in transportation of analytes to the bulk fluid. Langenfeld et al. (1994) used the kinetic models of Pawliszyn (1993) and Bartle et al. (1990) to explain the effect of modifiers on extraction, in order to "rationally optimise" the extraction.

Matrix: The matrix influenced the quantitative extraction significantly. This is not only due to the adsorption of analytes on the matrix, but also to several other mechanisms which limit the extraction. The analyte can be located or even trapped in interstitial micropores and hence a lengthy diffusion mechanism is required to reach the bulk fluid. The analyte may be covered by bulk organic material, which has to be extracted before the analyte can be recovered. The presence of water droplets can form a layer covering the absorbed solute, hence restricting access to the SCF for the interaction with the analyte.

Grinding of the sample often increases the recoveries due to creating a larger surface area, thereby allowing better diffusion of the solute from the sample matrix (Lee and Markides 1990). However, if the resultant particles are too small,

the pressure drop over the extraction cell increases and in certain circumstances the fine particles can cause restrictor plugging if the filters in the extraction cell are unable to retain the fine particles.

Water can either prevent the extraction of the analyte or it can assist the extraction by enhancing the solubility (Camel et al. 1993). One of the main concerns with the presence of water during the extraction is that it can cause restriction plugging due to freezing when the fluid is decompressed, which can only be avoided by careful restrictor heating.

Drying the sample before supercritical fluid extraction would circumvent this problem, however it has been shown to result in loss of volatile and semi-volatile compounds and thus cannot be used, if quantitative recovery of these were desired. Burford et al. (1993) therefore conducted a systematic study to evaluate 21 drying agents regarding their ability to retain water in the extraction cell. The presence of modifier caused less water to be retained, however restrictor plugging was only avoided with certain drying agents. Moreover, Burford et al. (1993) investigated the tendency of the drying agents to retain analytes, when dry samples were extracted. Hydromatrix was found to be the only drying agent not to adsorb solutes.

Derivatisation: Derivatisation reagents were used by Hills et al. (1991) in order to test if quantitative extraction had been achieved and it was noted that the addition of a derivatisation reagent assisted the extraction. This was either achieved by derivatising polar moieties of the solute, making it more soluble in the relatively non polar CO_2 or by derivatisation of active sites in order to prevent re-adsorption of the solute. Hawthorne's group investigated the use of trimethylphenylammonium hydroxide and boron trifluoride for the derivatisation of sulfonated aliphatic and aromatic surfactants in sewage sludge (Field et al. 1992), chlorinated pesticides (Hawthorne et al. 1992a, b), microbial phospholipids and phenol contaminated waste water with recoveries greater than 90% (Hawthorne et al. 1992a, b).

Optimisation: One of the greatest advantages of SFE is the choice of various parameters to influence the extraction recovery, however concomitantly this choice makes it difficult for the novice to decide on the parameters to start the optimisation. Gere and Derrico (1994a, b) outlined and discussed the first principles of method development, however the optimisation was performed on spiked samples. The same procedure was used by various authors (Lopez-Avila et al. 1990, van der Velde et al. 1994a), however van der Velde (1994b) reported later that conditions optimised for spiked samples had to be altered to achieve quantitative recoveries and hence should not be used for optimisation experiments unless they mimic the matrix and interactions accurately. There are numerous approaches for optimising extractions and one of the most practical and viable is that outlined by Hawthorne et al. (1993).

1.8 Aims and objectives of the project

The main objectives of this projects were to develop applications for supercritical fluid chromatography and extraction in pharmaceutical and environmental analysis. During the method development, knowledge should also be gained about the robustness and possible limitation of this technique.

The elution behaviour of four relatively polar alkaloids (nicotine, cotinine, anabasine and nornicotine), having different degrees of basicity, were studied on four different columns, namely (S)-NEC-β-CD, β-CD, diol and a silica column so as to investigate the limitation of the technique to elute basic compounds, which were likely to have limited solubility, react with residual silanols and possibly with the mobile phase. The influence of modifier concentration, pressure, temperature and additives was to be investigated and the use of

different amine additives to identify the most efficient additive to suppress ionisation and interactions with the residual silanols. The reproducibility of the retention times was used to assess if the retention mechanism was controlled, as variance in retention times would highlight the presence of more than one retention mechanism.

Since SFC is considered to be suitable for the separation of relative non polar to medium polar compounds, it was further envisaged to develop a method which would allow the separation of PCBs according to their toxicity. The separation of PCBs according to toxicity is usually performed using a normal phase technique with a hydrocarbon like hexane as a solvent. Therefore, a successful SFC method would result in reduction of solvent cost. For this the (S)-NEC-β-CD column was chosen, since the naphthyl group allows the formation of a electron-donor-acceptor complex.

Additionally, since PCBs accumulate preferentially in the adipose tissue of mammals, the separation of the PCBs from fats was investigated by using a suppository base to mimic human adipose tissue. The four columns used in the alkaloid separation were used to optimise the separation in terms of achieving the fastest separation.

PAHs are present in oils only in trace quantities due to their formation during high temperature usage of the oils. A separation of PAHs from oils was therefore investigated on the four columns, since the low levels of PAHs are difficult to quantify in the presence of large amounts of oils.

The applicability of SFC for chiral separations of phenethylamines, propanolol and clofibrate analogues was explored using a (S)-NEC-β-CD and β-CD column. The influence of parameters such as modifier and additives concentration, pressure and temperature were studied to find the most efficient separations and make recommendations on which parameters should be

preferentially changed in order to obtain fast separation without loss of chiral resolution.

The applicability of SFE was tested for the extraction of fatty acids from soya and cotton seed meal using a statistical design optimisation program. This was considered helpful for the novice to optimise the parameters for achieving an efficient extraction, as the number of parameters such as modifier type and concentration, temperature, flow rate and pressure are higher and their interdependence are more complicated than in normal liquid extraction.

Finally, the extraction of nicotine from tobacco was investigated, in which the following parameters were taken into account: influence of modifier type and concentration, particle size, water content, cell geometry, packing of the cell, flow rate and pressure.

CHAPTER 2

EXPERIMENTAL

2.1 Analytes and Chemicals Employed

All materials were used as received unless stated otherwise. Carbon dioxide (industrial grade) was obtained from BOC (Gilford, UK) with a purity of 99.8% and applied for all trials.

2.1.1 Alkaloid Separation

The alkaloids nicotine, cotinine, nornicotine and anabasine were purchased from Aldrich (Gillingham, UK), as were iso-propylamine (IPA), n-propylamine (NPA), butylamine (BTA), hexylamine (HXA), octylamine (OCA), triethylamine (TEA) and diethylamine (DEA). HPLC grade acetonitrile (MeCN), methanol (MeOH), i-propanol (2-PrOH) were all obtained from FSA (Loughborough, UK).

2.1.2 Polychlorinated Biphenyls (PCBs)

All PCBs were obtained from Greyhound/Chemservice (Birkenhead, Merseyside, UK).

2.1.3 Polyaromatic Hydrocarbons (PAHs)

A solution of the EPA 16 priority PAHs standard with a concentration of 100μg/ml was purchased from West Chester (PA, US).

2.1.4 Fats

Witepsol S55 from Brome & Schimmer Ltd. was kindly provided by Chris Koy of the dispensary, School of Pharmacy, Bath University.

Sunflower oil was purchased from Sainsbury (Bath, UK).

2.1.5 Phenethylamines

All nine racemic phenethylamines were supplied, via the Pharmazeutisches Institut der Universität Bonn (Germany), by Albert Roussel Pharma GmbH, Mauver Pharma GmbH, Boehringer Ingelheim, Hoechst AG and Glaxo GmbH.

2.1.6 Propranolol Analogues.

Racemic samples of propranolol and five of its analogues as HCl salts were kindly provided by Dr. G. Bedford of Zeneca Pharmaceuticals Ltd (Macclesfield, UK).

2.1.7 Fatty Acids Extraction

Fatty acids were purchased from Aldrich (Gillingham, UK): myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) acids. Methanol, 2-propanol and chloroform (all HPLC grade) were obtained from Fisons (Loughborough, UK).

α-Bromoacetophenone and triethylamine were purchased from Aldrich and fibrous Cellulose from Whatman Bio-Systems (Maidstone, UK).

2.1.8 Nicotine Extraction

Nicotine was purchased from Aldrich (Gillingham, UK). Methanol, 2-propanol, acetone and acetonitrile (all HPLC grade) were obtained from Fisons (Loughborough, UK). Dark shag tobacco was purchased in Spain. α-cellulose was obtained from Sigma (Poole, UK).

2.2 SFC

2.2.1 Columns Employed

The (S)-NEC-β-CD column (250 x 2.0mm, 5μm) and β-CD column (250 x 2.0mm, 5μm) were kindly donated by Technicol (Cheshire, UK). A Silica column and diol column (250mm x 2.0mm, Spherex 5μm) were purchased from Phenomenex (Macclesfield, Cheshire) and the ODS column (250mm x 4.6mm Apex II) from Jones Chromatography Ltd (Mid Glamorgan, UK). A Rheodyne precolumn filter with a 1.5mm frit filter (0.5μl) was obtained from Supelco (Bellefonte, PA, USA) and installed between the injector and the column.

2.2.2 SFC Set-up

All experiments were performed using a Jasco SFE system kindly donated by Jasco UK Ltd (Great Dunmow, UK) fitted with two pumps to allow the use of modifier. An ethylene glycol/water mixture was used with a cooler to maintain the head of the carbon dioxide pump model 980-PU at -10 °C. The outlet of the CO_2 pump was connected to channel A on the Jasco pump mixer PU 880-30 and the modifier pump to inlet B. Before the premixed fluid entered the oven model 860-CO, there was a SSI shut off valve installed to allow the column to remain pressurised overnight. Next in line was a an empty preparative HPLC column containing a Teflon rod, which worked concomitantly as a pulse dampener, a mixer and heat exchanger. The completely mixed and temperature equilibrated fluid entered a Rheodyne valve (a Rheodyne 7413 with a 1µl internal loop for the alkaloid investigation and a Rheodyne 7520 micro injection valve with 0.5µl volume for the remaining experiments), where sample injection took place. The fluid then flowed then through the pre-column filter, the column and the high pressure UV detector 875-UV (Jasco) to a model 880-81 backpressure regulator, which maintained the system at a chosen pressure. The exit tubing of the backpressure regulator was dipped into an appropriate solvent to allow collection of the injected compounds. Chromatographic data were collected using a HP 3395 integrator (Hewlett Packard, Stockport, UK).

2.2.3 Solute and Solvent Preparation

All solutions were filtered through 0.45µm pre-column filters (Supelco, Bellefonte, US).

Alkaloids: A stock solution containing 10mg/ml was prepared in MeOH for further dilution. Using the 7413 injector with an internal loop of 1µl, the alkaloids

were diluted to 200-400µg/ml in order to obtain a good response at 254nm. For calibration curves, solutions containing 4mg/ml to 20µg/ml were prepared by diluting the stock solution to the desired concentration.

The amine solution in MeOH were prepared by adding the appropriate amount of amine directly into the MeOH. The solution was sonicated for 10 minutes before use.

PCB: Remains of solid PCBs were dissolved in iso-octane and the appropriate dilution was determined by injecting the stock solution.

PAH: The PAH solution was diluted 1:1 with MeOH.

Phenylethylamine, propanolol and clofibrate analogues: Solutions with concentrations of 0.4-0.5g/ml in MeOH were prepared for the SFC trials.

Fat: 0.75g Witepsol S55 was suspended in 2ml of heptane and after sonication 2ml of 2-PrOH was added. The solution was warmed slightly to enhance the solubility, making the filtration step easier.

A 40% solution of the sunflower oil was prepared in heptane.

2.2.4 Pump Performance Test

The pump outlet was closed off and the flow rate set to 0.5ml/min. The pump was started and pressurised to 380kg/cm^2, where the flow rate was switched to 0.2ml/min. The pressure steps from 400 to 500kg/cm^2 were recorded. At normal performance, the pump required about 35-40 steps and 3 pressure dips were observed during the pressurisation from 400 to 500kg/cm^2. The pressure was held for 10 minutes and the pressure loss during this time monitored. The seals were considered to be in a good condition, when pressure did not drop more than 70kg/cm^2 during the 10 minute period.

2.2.5 Determination of Chromatographic Parameters.

1. Retention factors, k, were calculated using the following;

$$k = (t_r - t_0)/t_0 \qquad \text{(eq. 2.1)}$$

where t_r is the analyte retention time and t_0 is the retention time for an unretained solute under the experimental conditions (generally calculated from the injection of solvent, which was used to dissolve the analytes).

2. Resolution, R_s. The degree of resolution can be calculated by;

$$R_s = 2\,(t_2 - t_1)/(w_{b1} + w_{b2}) \qquad \text{(eq. 2.2)}$$

where w_{b1} and w_{b2} are the baseline width of peak 1 and 2 repectively.

3. Selectivity, α. The selectivity between two peaks was calculated thus;

$$\alpha = k'_1 / k'_2 \qquad \text{(eq. 2.3)}$$

where k'_1 is the capacity factor for the least retained of two analytes.

4. Plate height, H, was calculated according to equation 2.4;

$$H = \frac{(t_r / w_b)^2}{16} * 25 * 10000 \qquad \text{eq. (2.4)}$$

where w_b is the peak width at the baseline.

5. Efficiency, N. The efficiency was calculated using equation 2.5

$$N = 16\left(\frac{t_r}{w_b}\right) \qquad \text{eq. (2.5)}$$

2.2.6 Calculation of Critical Parameters

The following equations were used to calculate critical parameters of CO_2 and MeOH mixtures. The equations were added to a Microsoft Excel data sheet to allow the calculation of the critical parameters.

Critical temperature according to Chueh and Prausnitz was

$$T_c = \sum_j \theta_j T_{cj} + \sum_i \sum_j \theta_i \theta_j \tau_{ij} \qquad \text{eq. (2.6)}$$

where T_c and T_{cj} are the critical temperatures of the mixture and the pure component respectively

$$\theta_j = \frac{y_j V_{cj}^{2/3}}{\sum_i y_i V_{ci}^{2/3}} \qquad \text{eq. (2.7)}$$

where θ_j is the surface fraction, $V_{cj}^{2/3}$ the critical volume of the component j, cubic meters per kilogram mole, y_j mole fraction of the component j and

$$\delta_T = \left| \frac{T_{ci} - T_{cj}}{T_{ci} + T_{cj}} \right| \qquad \text{eq. (2.8)}$$

$$\psi_T = A + B\delta_T + C\delta_T^2 + D\delta_T^3 + E\delta_T^4 \qquad \text{eq. (2.9)}$$

the coefficients for CO_2 containing systems where

A = -0.0953, B = 2.185, C = -33.985, D = 179.068, E = -264.522

$$\tau_{ij} = \frac{1}{2} \psi_T (T_{ci} + T_{cj}),\ i \neq j \qquad \text{eq. (2.10)}$$

for the temperature estimation according to Li:

$$T_c = \sum_j v_j T_{cj} \qquad \text{eq. (2.11)}$$

with the volume fraction v_j defined as

$$v_j = \frac{y_j V_{cj}}{\sum_1 y_i V_{ci}} \qquad \text{eq. (2.12)}$$

Critical pressure was calculated according to the method of Kreglewski-Kay and T_c was obtained from the above methods or the SF-Solver program. The critical pressure and temperature of the pure components as well as the molar volume at a reduced temperature of 0.6. The acentric factor ω is also required.

$$P_c = P^*\left[1+(5.808 + 4.93\omega)\left(\frac{T_c}{T^*} - 1\right)\right] \qquad \text{eq. (2.13)}$$

$$\omega_{12} = \frac{2}{\left(\frac{1}{\omega_1}+\frac{1}{\omega_2}\right)} \qquad \text{eq. (2.14)}$$

$$\omega = \omega_1\theta_1 + \omega_2\theta_2 + (2\omega_{12} - \omega_1 - \omega_2)\theta_1\theta_2 \qquad \text{eq. (2.15)}$$

$$T^*_{12} = \frac{2V^{*1/3}_{12}}{\frac{\sum V^{*1/3}_i}{T_{ci}}} \qquad \text{eq. (2.16)}$$

$$T^* = V^{*1/3}\left[\frac{T_{c1}\theta_1}{V^{*1/3}_1}+\frac{T_{c2}\theta_2}{V^{*1/3}_2}+\left(\frac{2\,T^*_{12}}{V^{*1/3}_{12}}-\frac{T_{c1}}{V^{*1/3}_1}-\frac{T_{c2}}{V^{*1/3}_2}\right)\theta_1\theta_2\right] \qquad \text{eq. (2.17)}$$

$$\theta_i = \frac{y_i V^{*2/3}_i}{\sum y_i V^{*2/3}_i} \qquad \text{eq. (2.18)}$$

$$V^*_{12} = \frac{1}{8}\left(V^{*1/3}_1 + V^{*1/3}_2\right)^3 \qquad \text{eq. (2.19)}$$

$$V^* = V_1^* y_1 + V_2^* y_2 + \left(2V_{12}^* - V_1^* - V_2^*\right) y_1 y_2 \qquad \text{eq. (2.20)}$$

$$P^* = \frac{T^*}{V^{*1/3}} \left[\frac{\sum P_{ci}\theta_i}{\sum \frac{(T_{ci}\theta_i)}{\left(V_i^{*1/3}\right)}} \right] \qquad \text{eq. (2.21)}$$

2.2.7 Calculation of %Change in k

The figures in table 7.1 represent an increase in retention in the given range of the parameters. Looking at e. g. the pressure range of 300-150kg/cm^2, the capacity factor increased by 52% when the value at 150kg/cm^2 which is higher than that at 300kg/cm^2 is used as 100% for the calculation. If then for example one of the capacity factors however decreases within this range, a negative value is given, as seen for anabasine with increasing temperature. In order to allow a better comparison, a second value was calculated for a negative trend, where the highest value was chosen as 100%.

2.2.8 GC Analysis of PCBs

All extracts were analysed by a Hewlett-Packard (Bracknell, UK) HP 5890B gas chromatograph with a electron capture detector on a 25m BPX5 (0.2 mm i. d., 0.22μm film thickness) capillary column (SGE, Milton Keynes, UK). An 1μl aliquot of each collected fraction was injected onto the column in the on-column mode. The injector temperature followed the oven temperature program. A temperature program was used for the analyses, commencing at 40°C for 1min, followed by ramps of 20°C/min to 150°C and then 2.5°C/min to 250°C, and a final temperature of 250°C for 20min. Helium was used as the carrier gas at a flow rate of 1.2ml/min,

resulting in a linear velocity of 33.5cm/s. The detector temperature was set to 350°C and 30ml/min nitrogen make-up gas. The detector outlet was connected to a vent.

2.3 SFE of Cotton Seed and Soya Meal

All experiments were conducted without the additional Jasco PU 880-30 mixer, however the Teflon filled HPLC column served as the mixer, dampener and heat exchanger. The CO_2 and modifier valves were combined with a high pressure Rheodyne valve.

2.3.1 Instruments

All experiments were performed using a Jasco SFE system (Jasco UK Ltd, Great Dunmow, UK). An ethylene glycol/water mixture-filled cooler was used to maintain the head of the carbon dioxide pump model 980-PU at -5°C. The extraction vessel with an internal volume of 10ml (Jasco, UK) was kept at a set temperature in a column oven model 860-CO. A model 880-81 backpressure regulator kept the entire system under a selected, constant backpressure. This backpressure was regulated by an electronic feedback regulator which is flow independent. A 6-port Rheodyne valve was installed in the place of an injection valve which enabled both dynamic and static extraction.

All derivatised extracts were analysed by high-performance liquid chromatography (HPLC) using a modular system consisting of a SP 8100 gradient pump (Spectra-Physics Analytical, Stone, UK) and a Model 3100 UV-absorbance detector (LDC/ Milton Roy, Stone, UK). Chromatographic data were collected using a HP 3395 integrator (Hewlett Packard, Stockport, UK). The "design expert" system version 4.02 was purchased from Q D Consulting (Herts, UK) and the ISCO "SF-solver" version 2.5.1 from Jones Chromatography (Mid Glamorgan, UK).

2.3.2 SFE Procedure

2g of meal were mixed with about 6g cellulose and packed into the 10ml extraction cell. This was topped up with more cellulose to minimise the void volume. Cellulose was used as filling material because it is porous and a weak adsorbent. The extraction cell was then tightened, connected to the SFE unit and left for 15 minutes for temperature equilibration in the oven. The pump delivering the cooled carbon dioxide was then switched on and the system was pressurised. After pressurisation, the modifier pump was switched on and the extraction was started. The fatty acids were collected in 3ml of a 2-PrOH : chloroform (1:1) mixture in a vial wrapped with aluminium foil to avoid exposure of the extracts to light. The vial was cooled in an ice-water bath. Following the extraction the contents in the receiver were transferred to a 5ml volumetric flask and made up to volume.

2.3.3 Fatty Acid Derivatisation

An aliquot of the extract was evaporated to dryness and derivatised with α-bromoacetophenone according to Hanis et al. (1988) to form the phenacyl esters. After derivatisation the solvents were evaporated under a stream of dry nitrogen and the derivatisation products were dissolved in methanol (1ml). The fatty acid standards were dissolved in analytical grade acetone and derivatised using the same procedure as above. The derivatised fatty acids were separated on a 4.6mm i.d. x 25cm Hichrom ODS 5μm column using a flow rate of 1.0ml/min and a methanol : water gradient. The pump was programmed with a linear gradient from 70 to 85% methanol in 10 minutes, followed by an increase to 100% over the next 45min. The values obtained for the individual free fatty acids were then added together to produce a value for total free fatty acids, simplifying subsequent optimisation calculations.

2.3.4 Liquid Extraction in Blender

Meal samples (5g) were defatted with petroleum ether (60-80°C fraction) by blending for 2min in a conventional Waring Lab blender (Fisons, Loughborough, UK) with a meal : solvent ratio of 1:40 (Marshall et al. 1991).

2.3.5 Liquid Extraction using an ultrasonic Bath

Meal samples (2g) were suspended in 10ml chloroform : MeOH (2:1), sonicated for 15min and filtered using a vacuum unit (Jones Chromatography, UK). This was repeated with 9ml of fresh solvent and sonicated for another 30 minutes. Both extracts were combined and made up to 20ml.

2.3.6 Time-dependent Extractions

The cottonseed meal was treated and extracted in the same way as described under supercritical fluid extraction, however samples were taken after defined time intervals. This was done by stopping both pumps, quickly exchanging collection vials and switching the pumps on again. Each extract was derivatised and analysed. The sum of all free fatty acids present in the original sample was calculated using the following equation (Bartle et al. 1990a):

$$m_0 = m_1 + m_2^{\wedge 2} / (m_2 - m_3) \qquad \text{(eq. 2.21)}$$

in which m_0 is the total amount of free fatty acids in the original sample; m_1 is the extracted amount after time t_1; m_2 and m_3 are amounts of fatty acids extracted in subsequent, equal time intervals t_2 and t_3. The extracted mass, m_1, should be taken from the non-exponential part of the plot $\ln(m/m_0)$ versus time, whereas m_2

and m_3 should be taken from the exponential part. This is important for the correct calculation of m_0 in equation 2.21. After m_0 is calculated, the plot of $\ln(m/m_0)$ versus time is plotted to ensure that the masses m_1, m_2 and m_3 are taken at the correct times. The value of m represents the mass of extractable fatty acids that remain in the matrix after a certain time.

Equation 2.21 is derived from the hot-ball model, which was used by Bartle et. al (1990a) to evaluate the effects of matrix shape, size variation and solublity limitation on dynamic extraction. The model makes 3 assumptions which, when fulfilled result in complete conformity of the extraction behaviour with the model. First, the particles of the matrix should be spherical , the size of the particles should have a narrow size distribution and the analyte is assumed to be evenly distributed within the particles. Secondly, the flow rate is fast enough to ensure that the analyte concentration is zero at the particle's surface. Thirdly, the analyte moves through the matrix by diffusion. The resulting plot of $\ln(m/m_0)$ versus time of a dynamic extraction is characterised by a steep initial decline which is followed by an exponential decay whose slope is $1/t_c$ and is dependent on particle size and the diffusion coefficient, and an intercept I of a theoretical value of -0.49977. When real samples are extracted, the three assumptions are often not fulfilled and hence influence the theoretically derived plot. If the particle has an irregular shape and therefore a greater surface-to-volume ratio, the initial fall will be larger, although the slope remains the same. A smaller particle size, which can be attained by grinding, will allow faster extraction which can be observed by a larger slope. The grinding process presses the solute to the particle surface and thus yields a steeper initial fall and a longer time to establish a smooth concentration profile. The time, $0.5t_c$, is the time when the curve $\ln(m/m_0)$ versus time becomes linear and a smooth concentration profile is established. Another deviation of the theoretical curve can occur when the extraction is solubility limited. This reduces the initial rate of the extraction and therefore delays the establishment of the linear portion.

2.4 SFE of tobacco

2.4.1 Instrumental Set-up

SFE set-up as described in 2.3.1.

All extracts were analysed by high-performance liquid chromatography (HPLC) using a modular system consisting of a SP 8100 gradient pump (Spectra-Physics Analytical, Stone, UK) and a Model 3100 UV-absorbance detector (LDC/ Milton Roy, Stone, UK). Chromatographic data was collected using a HP 3395 integrator (Hewlett Packard, Stockport, UK).

The tobacco was powdered using an electric mill M20 IKA-universal mill (Sartorious, Epsom, UK)

The powdered tobacco was sieved on a Fritsch 'analysette' type 03.502 (Christison Scientific Equip. Ltd., Gateshead, UK) using test sieves (Endocotts Ltd., London, UK) with a diameter of 20cm and the following mesh sizes: 355μm, 250μm, 180μm and 125μm.

2.4.2 Preparation of the Tobacco

Fresh tobacco was loosely distributed in the electric mill and powdered twice for 1 minute. About 25g powdered tobacco were then transferred onto the top sieve (355μm) and attached onto the Fritsch 'analysette' and sieved for 15 minutes at an amplitude of 3. The sieves were then emptied, cleaned gently with a brush and the

tobacco fractions returned to the appropriate sieve. The sieving process was continued for another 15 minutes.

The water content of the powdered tobacco fractions was determined by drying the fractions in an oven at 60°C for 24 hours to a constant dry weight. Table 2.1 lists the water content of the fractions as used for the SFE and their nicotine content, which was determined by time-dependent extractions using the hot-ball model equation 2.21.

Table 2.1 Levels of water in different tobacco fractions

Tobacco Fraction [μm]	Fraction Number	Water [%]
125 - 180	1	10.0
180 - 250	2a	12.4
180 - 250	2b[b]	0
250 - 355	3	14.9

[a] determined by SFE, level calculated on dry weight basis
[b] fraction 2b was dried at 60°C for 24 hours

2.4.3 SFE Procedure

Packing 1: First 1.5g α-cellulose was packed into the 10ml extraction cell, then 0.5g tobacco were thoroughly mixed with 1.0g α-cellulose and packed into the cell. This was topped up with 1.0g α-cellulose to minimise the void volume. The extraction cell was then tightened, connected vertically into the SFE unit, so that the 1.5g α-cellulose was on top and left for 10 minutes for temperature equilibration in the oven. The pump delivering the cooled carbon dioxide was then switched on and the system pressurised. After pressurisation, the modifier pump was switched on and the extraction was started. Nicotine was collected in 5ml methanol in a vial which was wrapped with aluminium foil to avoid exposure of the extracts to light. The vial was cooled in an ice-water bath. Following the

extraction the content in the receiver was transferred to a 20ml volumetric flask and made up to volume with methanol. The extracts were diluted with mobile phase and filtered using a 0.45μm nylon filter before HPLC analysis.

Packing 2: 3.5g α-cellulose was packed into the 10ml extraction cell and 0.5g tobacco was added. The extraction cell was connected to the SFE unit as before, so that the 3.5g cellulose was at the top. The packing of the cell required approximately 10 minutes.

The packing of the 1.67ml cell was easier as only 0.5g tobacco were filled into the cell.

The extracts were separated on a 4.6mm i. d. * 25cm Hichrom ODS 5μm column using the method of Zuccaro et al. (1993). The nicotine levels were calculated on dry weight basis.

2.4.4 Gas-Chromatography and Identification

The tobacco extracts were analysed on an HP 5890/5970C GC-MS system (Hewlett Packard, Stockport, UK). Hydrogen was used as carrier gas (flow rate 1ml/min) and the injector and transfer line was kept at 250°C and 280°C respectively. The chromatographic separation was performed on a 50m DB-5 bonded phase (0.22mm i. d., 0.25μm thickness,) capillary column (SGE, Milton Keynes, UK). The initial temperature was kept at 60°C for 2 minutes and was raised at increments of 10°C/min to 150°C, held for 10 minutes. This was followed by a further increase of 5°C/min to a temperature of 200°C, which was held for 10 minutes. A final increase of 5°C/min brought the temperature to 275°C.

The peaks were identified using the library search facility.

2.4.5 Collection Efficiency

The extraction cell was completely filled with α-cellulose and extracted for 10 minutes using the following conditions: 8mol% methanol, 50°C, 200kg/cm^2 and 3ml/min total flow rate. The cell was disconnected, opened and spiked with 100µl of approximately 0.2g/ml nicotine standard, which correlates to the quantity present in 1g tobacco. The nicotine was then extracted for 30 minutes using different amounts of methanol as collection solvent.

2.4.6 Time-Dependent Extractions

Tobacco was packed into the cell in the same way as described under Supercritical Fluid Extraction; however samples were taken after the following time intervals: 5, 10, 20, 30, 60, 90, 120, and 150 minutes. This procedure has been described in 2.3.7.

2.4.7 Liquid Extraction.

The liquid extraction was performed according to Saunders et al. (1981). Tobacco (0.5g) was extracted for 24 hours under constant agitation with 12ml of 25mM KH_2PO_4 buffer pH 7.8. The extract was filtered using a Buchner flask and funnel with a Whatman No 1 filter-paper. The extract was diluted for analysis. This lengthy extraction procedure was chosen to ensure that all the nicotine is extracted in order to provide a comparison for the SF extractions.

2.4.8 Accelerated Solvent Extraction

The Jasco SFE-unit was rebuilt to allow "accelerated solvent extraction"described by Richter et al. (1996). Tobacco (0.5g) was weighed into the 10ml extraction cell

and connected vertically into the SFE unit. 12ml of MeOH : 41.2mM KH_2P0_4 (2:3) was pumped into the cell, the temperature was set to 100°C and the backpressure regulator to 140kg/cm^2. The system was left for 5min to heat up and 10min for temperature equilibration. 10 minutes were then allowed for static extraction, which was followed by releasing the pressure by changing the setting on the backpressure regulator. The cell was flushed with 6ml new solvent-mixture and purged for 10 minutes using $C0_2$, before being flushed with another 6ml solvent and finally purged with $C0_2$ for 10 minutes. The extracts were transferred into a 50ml volumetric flask and made up to volume.

CHAPTER 3

SFC RESULTS AND DISCUSSION

3.1 Separation of Nicotine Alkaloids

The determination of nicotine is of particular importance in toxicological studies and also in the tobacco industry (Atkinson et al. 1984). For studies regarding the toxicology of nicotine, it is necessary to include metabolites and secondary metabolites of nicotine namely, cotinine and trans-3'-hydroxycotinine, cotinine N-oxide and norcotinine (Zuccaro et al. 1993). Nicotine is, with 98 % of the total alkaloid fraction, the major component in commercial flue-cured tobacco and the minor alkaloids are nornicotine, anabasine, anatabine and 2,3'-dipyridyl (von Euler 1965). These alkaloids contribute to the quality of tobacco and are therefore important for breeding programs in which it is useful to monitor and control alkaloids in tobacco (Saunders and Blume 1981). Furthermore, nicotine is prone to oxidation, which produces nicotinic acid, nicotyrine, cotinine and myosmine (Wada et al. 1959). Hubert-Bierre et al. (1975) identified oxidation products such as nicotyrine, cotinine and nicotine N-oxide after exposing a methanolic solution of nicotine to oxygen in the presence of methylene blue. They developed a sensitive method capable of separating and detecting the different alkaloids quantitatively at very low levels with inverse fluorescence detection.

3.1.1 Introduction

In general, HPLC has been used in the determination of nicotine and its major metabolite cotinine in urine (Watson 1977 and Kyerematen et al. 1982) and in

serum (Zuccaro et al. 1993, Kyerematen et al. 1987). Either normal-phase (Watson 1977) or reverse-phase chromatography (Zuccaro et al. 1993, Sudan et al. 1984, Saunders and Blume 1981) was used. Zuccaro et al. (1993) separated nicotine and four major metabolites as well as the internal standard N-ethylnorcotinine and caffeine, which is frequently present in the plasma of smokers. A conventional C_8-column was used, requiring the addition of ion-pair reagents and an analysis time of about 35 minutes plus a 15 minute equilibration time. Alternatively, a base deactivated column, which did not require the addition of an ion-pair reagent, achieved the separation in about 20 minutes and only 10 minutes equilibration time was required. Sudan et al. (1984) achieved the separation of nicotine and cotinine on a C_8-column with a methanol-buffer mobile phase within 8 minutes, however no selectivity or resolution was stated and the nicotine peak appeared slightly tailed. Tobacco related alkaloids such as anabasine, anatabine and nornicotine were separated from nicotine on an ODS-column using methanol containing phosphoric acid buffered with triethylamine. Good separation of nicotine from the other alkaloids was achieved, despite the tailing nicotine peak and nornicotine, anabasine and anabatine were not completely baseline resolved (Saunders and Blume 1981).

β-cyclodextrin (β-CD) has been successfully used in the separation of tobacco alkaloids in the reversed-phase mode by Seeman et al. (1989) and Armstrong et al. (1990a). Control of pH was vital for the separation and elution order, however no chiral separation was observed in these investigations.

Other methods of determining nicotine involve the use of circular dichroism spectropolarimetry (Atkinson et al. 1984), a rapid GC method (Severson et al. (1981) and a thermospray chromatographic-mass spectrometric method (McManus et al. 1990).

There has been no publication of tobacco alkaloids separated via SFC, however Janicot et al. (1988) reported the separation of opium alkaloids on a silica and an

aminopropyl column. They conducted a thorough investigation on the effect of methanol modifier and as this did not result in efficient resolution of the alkaloids, aliphatic amines such methylamine, ethylamine and triethylamine were added to enhance the separation. It was noted that the addition of water increased the retention on the aminopropyl column, however had little an effect on the selectivity on the silica column. Berry et al. (1986) investigated the use of SFC for the analysis of polar drugs and separated ergot alkaloids on an aminopropyl column using methanol as modifier, as problems were encountered on the silica column. The use of a basic modifier was not investigated due to the common perception that basic additives will form insoluble salts with CO_2 (Francis 1954 and Dandge et al. 1985).

Ashraf-Khorassani and Taylor (1988a) investigated the elution behaviour of nitrogen containing compounds using an aminopropyl-column with CO_2 as mobile phase and observed that the elution order neither corresponded to the elution order of the same compounds in the RP mode nor to that in the NP mode. They concluded that in SFC at lower density the retention mechanism depended on basicity, steric hindrance and on solubility of the solute in CO_2. At higher density the elution order was mainly influenced by basicity and steric hindrance. Fields and Grolimund (1988) investigated the limit of basicity of amines which could be eluted from a capillary column coated with a methylpolysiloxane using CO_2 as mobile phase. The limit of elution was to be a pK_a of 9 by Dandge et al. (1985) due to the formation of insoluble salts with amines having a pK_a above 9. This limit was confirmed for secondary alkylamines, however did not apply to tertiary amines (Fields and Grolimund 1988). Investigations have shown that this pK_a limit is lower on packed columns, which was attributed to reactive silanols causing peak tailing (Ashraf-Khorassani and Taylor 1988b). As a result, many researchers suggested the use of less polar stationary phases which are highly deactivated to circumvent peak tailing and irreversible adsorption (Ashraf-Khorassani and Taylor 1988b, Ashraf-Khorassani et al. 1989 and Lee and Markides 1986). Berger and Deye (1991e)

confirmed improved peak shapes for the elution of anilines and toluidines on a deactivated column, however when 1% MeOH was added the peak shapes on all columns improved. It was further stressed that the MeOH enhanced the solvent strength of the mobile phase and modified the stationary phase. Berger and Deye (1992) concluded that the addition of modifier caused non-linear retention factor changes which were due to solvent strength variation and independent of surface phenomena, however it was further stressed that at modifier concentrations below 1-2%, interactions with silanols were possible.

The separation of nicotine alkaloids such as cotinine, nornicotine and anabasine on a (S)-naphthylethyl carbamate derivatised β-cyclodextrin (S)-NEC-β-CD, β-cyclodextrin (β-CD), diol and silica was investigated. Since all of the alkaloids are chiral compounds both enantiomers were investigated to see whether chiral resolution was feasible using chiral columns. Additionally, it was of interest to see whether the compounds can be eluted from any of the columns without the addition of basic additive, so that a NPD could be used for determining trace levels of the alkaloids.

3.1.2 Calculation of Critical Parameters

Since solute retention in SCF is governed by solute interactions in the stationary and mobile phase and volatility (at high temperatures), the mobile phase has to be fairly polar for polar compounds (Berger and Deye 1991b). However SC-CO_2 is only a weak Lewis base and not able to elute polar compounds (Lee and Markides 1990), hence it is necessary to add a polar modifier (entrainer, co-solvent) to enhance the fluid's polarity. As reported in the separation of ergot alkaloids (Berry et al. 1986) and opium alkaloids (Janicot et al. 1988) the addition of a modifier was essential for elution and the additional additive improved the separation (Janicot et al. 1988). Crowther and Henion (1985) investigated the use of SFC-MS for the separation of polar drugs and stressed the importance of performing separations in

the supercritical region in order to maintain high efficiency levels. Critical temperature and pressure was calculated according to Chueh and Prausnitz (1967) and Kreglewski and Kay (1969) respectively, as Spencer et al. (1973) recommended these as being the most accurate.

Mixed binary mobile phases possess different phase behaviour (Chapter 1.3.1), and it is essential to avoid the vapour-liquid equilibration region in order to obtain useful results (Berger and Deye 1991b). The critical temperature and pressure of a mixture of CO_2 and MeOH were calculated as described in the Experimental 2.2.6 to ensure that the separation of the tobacco alkaloids were conducted in a one-phase region, in order to avoid baseline disturbances due to phase separation.

For the calculation of critical temperature the approaches of Chueh and Prausnitz (1967) and Li (1971 cited in Reid et al. 1987) were used, and for the critical pressures the approach Kreglewski and Kay (1969). The calculation of critical temperature and pressure was summarised and demonstrated on binary mixtures by Reid et al. (1987) and Danner and Daubert (1983) to assist the procedure. The calculation of critical pressure relied on the critical temperature, and therefore different critical pressures were obtained when using different critical temperatures calculated from the above methods and from the SF-Solver™ (Isco 1991). The critical parameters were calculated over a MeOH concentration range of 1 - 50% in CO_2. Table 3.1 shows the values obtained for critical temperature and for comparison the values calculated with the SF-Solver have also been included. The MeOH concentrations in Table 3.1 are also given in mole fractions in % to make a comparison with published values possible. The v/v% were transformed to mole fractions in % by assuming that the density of CO_2 was 1.039g/ml at -5°C and 200kg/cm^2 (L'Air Liquide) and the MeOH density was about 0.791g/ml at room temperature (Baker HPLC Manual).

Table 3.1 Critical temperatures for MeOH and CO_2 mixtures using different approaches

MeOH conc. [%v/v]	MeOH conc. [% mole fraction]	T_c Li [°C]	T_c Chueh [°C]	T_c SF-solver [°C]
1.0	1.0	33.52	34.53	33.10
3.0	3.1	38.54	41.49	37.40
5.0	5.2	43.52	48.29	41.70
10.0	10.4	55.79	64.63	52.40
15.0	15.6	67.80	80.07	63.10
20.0	20.7	79.56	94.65	73.70
25.0	25.8	91.09	108.41	84.30
30.0	30.9	102.38	121.39	94.90
40.0	41.1	124.29	145.16	116.20
45.0	46.1	134.93	156.03	126.60
50.0	51.1	145.36	166.26	137.10

There are few publications of critical parameters of mixed fluid mixtures, which made a comparison difficult, however Brunner et al. (1987) investigated the isothermal phase equilibria of CO_2 and MeOH mixtures and observed three critical points. These are listed in Table 3.2.

Table 3.2 Critical points of binary mixtures of MeOH and CO_2[a]

MeOH [w/w%]	T_c [°C]	P_c [kg/cm^2]
15.96	50	93.65
32.65	100	151.22
48.22	150	158.18

[a] values taken from Brunner et al. (1987).

When comparing the values for the critical points in Table 3.1 and 3.2 it can be seen that at 15% MeOH the method of Li and the SF-Solver overestimated the values whilst the approach of Chueh and Prausnitz resulted in considerable

deviation from those of Brunner et al. (1987). At around 32% MeOH the SF-Solver and the method of Li gave the most similar results, however at 48% MeOH the method of Chueh and Prausnitz concurred closest with the published values. Table 3.3 lists the calculated values for the critical pressure using the critical temperatures obtained from Li, Chueh and Prausnitz and from SF-Solver.

Table 3.3 Critical pressures obtained for CO_2 and MeOH mixtures using different critical temperatures

MeOH conc. [%v/v]	MeOH conc. [%w/w]	P_c [kg/cm^2] with T_c Li	P_c [kg/cm^2] with T_c Chueh	P_c [kg/cm^2] with T_c SF-solver
1.0	1.0	73.45	75.16	72.74
3.0	3.1	75.58	80.54	73.66
5.0	5.2	77.58	85.52	74.54
10.0	10.4	81.99	96.4	76.46
15.0	15.6	85.65	105.25	78.14
20.0	20.7	88.63	112.28	79.44
25.0	25.8	91.02	117.69	80.56
30.0	30.9	92.85	121.65	81.51
40.0	41.1	95.05	125.77	83.13
45.0	46.1	95.49	126.16	83.39
50.0	51.1	95.55	125.56	83.69

The values for the critical pressure show clearly how the calculated values of critical pressure depended on the choice of the critical temperature and the severe discrepancies from the results obtained by Brunner et al. (1987). However, the accuracy of these values were sufficient to avoid phase separations, since it was only necessary to be above either the critical pressure or temperature to avoid phase separation (Berger and Deye 1991b). Page et al. (1991a) used the above methods and additional approaches for calculating critical parameters of CO_2 and MeOH and found that these deviate considerably from experimental values. However, the critical pressures listed by Page et al. (1991a) as experimental determined values were considerably different to the one listed by Berger and

Deye (1991b) and by Brunner et al. (1987). Page et al. (1991a) reported a 16% MeOH mixture in CO_2 related to a critical pressure of about 145kg/cm^2, whereas Berger and Deye (1991b) listed it at around 94kg/cm^2. The calculated values in Table 3.3, however, are in close agreement with the values calculated by Page et al. (1991a).

Berger and Deye (1991b) observed no phase separation when either temperature or pressure were chosen below the given critical parameters, however baseline disturbances were noted when the vapour-liquid equilibrium zone was crossed, since phase separation occurred. Berger (1993) further recommended near critical pressure conditions to be avoided due to the presence of a thick layer of adsorbed mobile phase on the stationary phase as this layer seemed to be rather unstable and caused irreproducible results.

As it was envisaged to investigate the retention behaviour with temperature in the range of 40-100°C, the pressure should be at least 150kg/cm^2 to avoid phase separation and the presence of a thick layer of adsorbed mobile phase.

3.1.3 Irreproducible Retention Times

During the initial investigation using the (S)-NEC-β-CD column it was noted that the retention times were continuously decreasing and could have represented a degradation of the stationary phase. Since it was not clear what caused the significant decrease in retention times, the column was thoroughly rinsed and the β-CD and diol column were used to investigate the problem, as these columns were only a fraction of the cost of the (S)-NEC-β-CD.

The β-CD column was equilibrated for 10 minutes at 15% MeOH containing 0.5% DEA, at 1.0ml/min, 40°C and 200kg/cm^2. The elution order of the alkaloids was nicotine, cotinine, nornicotine and anabasine. Since the retention times were changing over several hours, the column was left to equilibrate overnight at the

same conditions at a flow rate of 0.5ml/min. The retention times reduced as indicated in Table 3.4 and was most significantly for anabasine, as seen in the calculated %change.

Table 3.4 Variation of retention times [min] of alkaloids in a non-equilibrated column

Time elapsed	Retention time [min]			
	Nicotine	Cotinine	Nornicotine	Anabasine
before overnight equilibration	1.463	1.666	3.657	4.710
after overnight change[a]	1.369 (6.4%)	1.584 (4.9%)	3.384 (7.5%)	4.230 (10.2%)
after 2 hours at conditions below[a]	1.360 (7.0%)	1.573 (5.6%)	3.346 (8.5%)	4.177 (11.3%)
after 5 hours at conditions below[a]	1.354 (7.5%)	1.576 (5.4%)	3.366 (8.0%)	4.155 (11.8%)

Conditions: β-CD column, 1.0ml/min, 40°C, 15% MeOH + 0.5% DEA, 200 kg/cm^2.
[a] % expressed on the basis of the run before the overnight equilibration.

Even though the system was equilibrated overnight the retention times of anabasine continued to decrease, which can be seen in Figure 3.1, depicting the retention time differences of anabasine-nornicotine and cotinine-nicotine. Since only the retention of anabasine decreased significantly, the difference in retention time between anabasine-nornicotine was decreasing. Even after 5 hours of equilibration time and continuous injection every 15 minutes it was impossible to achieve a constant retention time for anabasine.

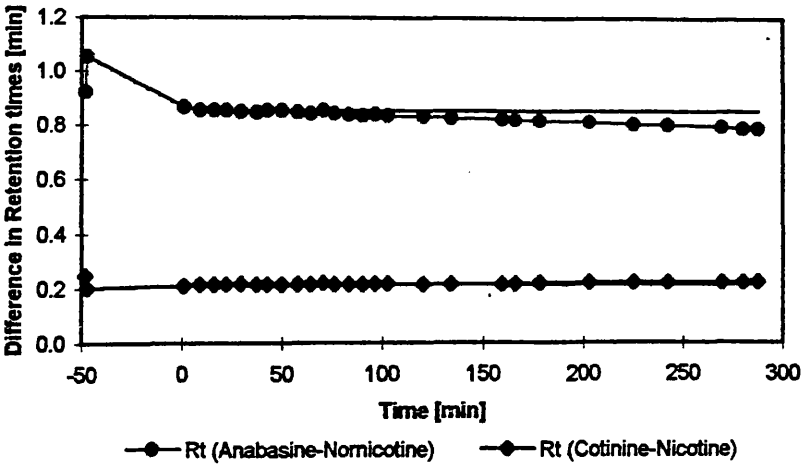


Figure 3.1 Difference in retention times between the alkaloids.
Additional line shows the continuation of the initial value.
Conditions: β-CD column, 1.0 ml/min, 40°C, 15% MeOH + 0.5% DEA, 200 kg/cm^2.

Moreover, since the analysis of p-nitroaniline and m-nitroaniline resulted in constant retention times, it was concluded that the column had not degraded, but the separation of the alkaloids must be influenced by additional parameters.

Addition of H_2O: The slow decrease in the retention times of the alkaloids, when equilibration takes place at low temperatures, and the more severe change in retention times when the column temperature was raised between investigations at low temperatures were considered to be associated with absorption of water in the stationary phase. Water solubility in CO_2 increases with temperature (Coan and King 1971) and additionally the interaction between the stationary phase and water decreases with temperature. Addition of water should therefore increase the retention times and the resolution of nornicotine and anabasine. In a trial, in which 5% water was added to the mobile phase (conditions otherwise as in Figure 3.1), the retention of all the alkaloids increased, although to different extents. Nicotine retention increased by 21%, cotinine by 18%, nornicotine by 22% and anabasine by 51% upon water addition. This behaviour resulted in a decrease in the difference in retention times between cotinine-nicotine and an increase between anabasine-nornicotine as visualised in Figure 3.2.

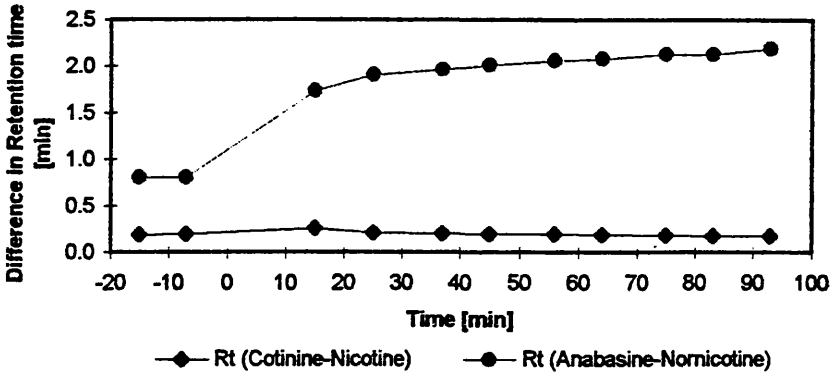


Figure 3.2 Difference in retention times between the alkaloids.
Conditions: β-CD column, 1.0ml/min flow rate, 40°C,
15% MeOH + 5% H_2O + 0.5% DEA, 200 kg/cm^2.

In order to release the water again, the column was equilibrated at 70°C for 1 hour, however equilibration was not achieved. The column was then kept at 100°C and at conditions as in Table 3.4 to equilibrate overnight. According to Technicol (1995) the CD columns are stable up to 180°C as they were derivatised at these temperatures. It was interesting to observe that anabasine eluted last when water was present in the stationary phase, however it eluted before nornicotine after the column had been equilibrated overnight to remove all the water. Figure 3.3 shows the change in retention time after 1 hour at 70°C and after the overnight equilibration at 100°C.

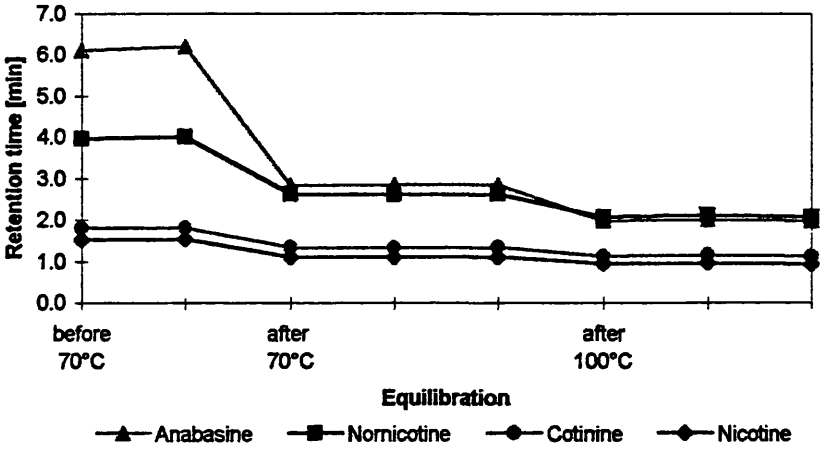


Figure 3.3 Change in elution order after column conditioning.
Conditions as in Figure 3.2.

The retention times of nicotine and cotinine changed only marginally compared to the changes in anabasine and nornicotine, however resolution was enhanced between both pairs when water was present. However when water was used in the mobile phase, the system took substantially longer to equilibrate. On columns conditioned so that insignificant amounts of water are present, nornicotine eluted last at 25°C. Janicot et al. (1988) also observed longer retention times and better resolution in the presence of water.

This peak reversal was only observed at 40°C, since at higher temperatures anabasine eluted considerably earlier than nornicotine with or without the presence of water in the mobile phase. The elution order observed by Armstrong et al. (1990a) on a β-CD column using HPLC with MeOH as modifier at a pH of 5.5 was nornicotine, anabasine, nicotine and cotinine at room temperature. When the results of Armstrong et al. (1990) were compared to the results obtained using SFC at 25°C the alkaloids eluted almost in reverse order to that found in HPLC, namely of nicotine, cotinine, nornicotine and anabasine. The reversal in elution order must have been caused primarily by solubility effects and to a lesser extent by the different retention mechanism on the β-CD column, when using it in SFC instead of the reverse-phase mode, in which the strength of the inclusion complexes seem to determine the elution order. For more detailed information and discussions regarding the structure and retention mechanisms using CD columns, see chapter 4.

Water can be introduced also involuntarily by the modifier which is not absolutely dry. It was observed, that despite using dry MeOH and amine additives, the retention time of the alkaloids increased over a period of 2 days when using the same amine solution, however the original retention can be restored by heating the column. The problem could be alleviated by installing a syringe filled with drying material into one of the two pre-drilled holes in the screw top of the modifier reservoir. The tubing supplying the modifier to the pump was connected through the second hole and both holes were sealed with Teflon tape to prevent air being

drawn into the modifier reservoir. The air drawn into the bottle by the liquid withdrawal was therefore dried and hence eliminated the possibility of moisture introduction from the incoming air. Figure 3.4 shows the syringe filled with drying material.

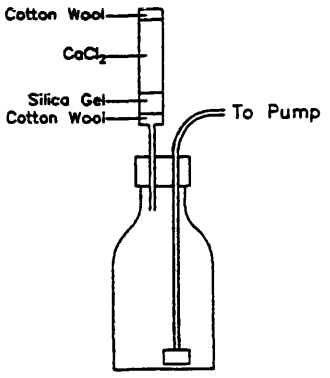


Figure 3.4 Drying syringe installed onto the modifier reservoir.

Table 3.5 shows the results obtained before and after the installation of the drying syringe.

Table 3.5 Variation in retention factor and resolution due changing H_2O content in the modifier

Coditions	k Nic	k Cot	k Ana	k Nor	Rs (Nic-Cot)	Rs (Cot-Ana)	Rs (Ana-Nor)
new iso-PA solution without drying syringe	0.315	0.883	1.535	3.457	5.121	4.475	8.654
old iso-PA solution after overnight run without drying syringe	0.349	0.937	1.748	3.761	5.332	5.456	8.558
new iso-PA solution after overnight run without drying syringe	0.343	0.926	1.690	3.673	5.266	5.320	8.909
new iso-PA after 30 minutes conditioning without drying syringe	0.320	0.889	1.559	3.493	5.113	4.776	8.897
new iso-PA solution with drying syringe (100°C equilibration before)	0.228	0.694	1.151	2.807	4.509	3.621	8.890
old iso-PA solution after overnight run with drying syringe	0.222	0.688	1.129	2.802	4.476	3.464	8.918

Conditions: Diol column, 0.5ml/min, 50°C, 8% MeOH + 1.025% iso-PA, 200kg/cm^2.

The results on the diol column showed clearly that retention times increased with the presence of water, however to a different extent than on the β-CD column in Figure 3.2 as the retention times were investigated at 40°C. At higher temperatures the addition of water caused an increase in retention factors for all alkaloids and an increase in nicotine-cotinine and cotinine-anabasine resolution, whereas the resolution of anabasine-nornicotine decreased slightly.

However, in spite of controlling the presence of water, the retention times still showed considerable day-to-day variations. The influence of varying the flow rate of CO_2 at constant modifier flow rate was investigated in an attempt to explain the variation in the results. It was noted that t_0 decreased over a day which must have been caused by an increase in the total flow rate. The reason for this could be improvement of pumping efficiency due to expanding pump seals or due to changing temperature in the pump head. A decreasing temperature of the pump head would increase the density of CO_2 in the pump head and since constant flow rates were delivered, more CO_2 would have been delivered.

Increasing the CO_2 flow rate and keeping the modifier flow rate constant resulted in a decrease in retention time of nicotine, cotinine and anabasine, and an increase in nornicotine retention. This resulted in an almost unchanged retention factor of nicotine and an increase in the cotinine, anabasine and nornicotine retention factor due to relative changes compared to the dead time marker. Resolution and selectivity of all the alkaloid pairs increased as shown in Table 3.6.

Table 3.6 Variation in retention factor and resolution due to change in CO_2 flow rate

Conditions	k Nic	k Cot	k Ana	kNor	Rs (Nic-Cot)	Rs (Cot-Ana)	Rs (Ana-Nor)
0.85ml/min CO_2, 0.15ml/min modifier	0.377	0.790	1.476	2.600	3.000	4.411	5.086
0.90 ml/min CO_2, 0.15 ml/min modifier	0.378	0.810	1.518	2.727	3.093	4.492	5.333
% change	0.3	2.5	2.9	8.6	3.1	1.8	4.9

Conditions: β-CD column, 1.0ml/min, 60°C, 15% MeOH + 0.75% DEA, 200kg/cm^2.

The same phenomenon was observed when the temperature in the laboratory rose. It was assumed that the increasing head pressure of the CO_2 cylinder increased the CO_2 density in the pump head, thus causing an increased flow of CO_2 when the temperature rose in the laboratory. This was confirmed by heating the cylinder with an electrical heater and analysing the alkaloids as shown in Table 3.7.

Table 3.7 Variation in retention factor and resolution due to changing temperature of the CO_2 cylinder

Coditions	k Nic	k Cot	k Ana	k Nor	Rs (Nic-Cot)	Rs (Cot-Ana)	Rs (Ana-Nor)
cylinder at room temperature	0.322	0.652	1.201	2.206	2.547	3.769	5.204
heater on cylinder	0.330	0.677	1.240	2.313	2.604	3.823	5.363
% change	2.5	3.8	3.2	4.9	2.2	1.4	4.2

Conditions: β-CD column, 1.0ml/min, 60°C, 12% MeOH + 0.615% iso-PA, 200kg/cm^2.

In order to circumvent this problem of irreproducible heating of the cylinder a braced PVC tubing (about 60m) was wrapped around a wooden cage and connected to a water bath set to 30°C. The outlet of the water bath was connected to the tubing on the bottom of the cylinder so as to heat up the liquid CO_2 in the cylinder. A major drawback of this set up was the relatively low flow rate of the water pump, resulting in the water taking at least 5 minutes to travel through the tubing, hence the temperature could not be kept completely constant. It can be argued that the heating of the cylinder actually decreased the density and should have resulted in a lower flow rate. However, as the CO_2 is isobarically cooled from the cylinder to the pump and in the pump, the CO_2 possessed a greater density in the pump head and thus more CO_2 was delivered.

The same effect was obtained by setting the cooling bath from -7.5°C to -10°C as seen in Table 3.8 from the variation of the retention factor and resolution.

Table 3.8 Variation in retention factor and resolution due to changing temperature of the CO_2 pump head

Conditions	k Nic	k Cot	k Ana	k Nor	Rs (Nic-Cot)	Rs (Cot-Ana)	Rs (Ana-Nor)
pump head at -7.5°C	0.208	0.643	1.062	2.609	4.894	3.711	8.890
pump head at -10°C	0.209	0.656	1.091	2.726	4.923	3.762	9.192
% change	0.5	2.0	2.7	4.5	0.6	1.4	3.4

Conditions: Diol column, 0.5ml/min, 50°C, 8% MeOH + 1.025% iso-PA, 200kg/cm^2.

The relative changes given in Table 3.6 to 3.8 did not change in relation due to the different conditions used, however the same trend was observed in each investigation.

Another parameter in influencing the retention times of the alkaloids was the temperature of the modifier pump, since severe temperature variations of 18-30°C occurred in the laboratory. These could have had an additional effect on the separation, in particular as small variations in modifier had a relatively large effect on the retention of the alkaloids as seen in Table 3.9.

Table 3.9 Variation in retention factor and resolution due to change in MeOH flow rate

Conditions	k Nic	k Cot	k Ana	k Nor	Rs (Nic-Cot)	Rs (Cot-Ana)	Rs (Ana-Nor)
0.85 ml/min CO_2, 0.15 ml/min modifier	0.377	0.790	1.476	2.600	1.500	2.206	2.543
0.85 ml/min CO_2, 0.14ml/min modifier	0.384	0.829	1.555	2.815	1.671	2.415	2.903
% change	1.9	4.9	5.4	8.3	11.4	9.5	14.2

Conditions: β-CD column, 1.0ml/min, 60°C, 15% MeOH + 0.75% DEA, 200kg/cm^2.

Due to the decreasing solvent strength and the lower total flow rate, retention and resolution increased with decreasing modifier concentrations. The same effect should be produced by heating up the modifier pump head, which was achieved by installing a temperature controlled clamp-on head onto the modifier pump. Table 3.10 represents the results obtained for the separation.

Table 3.10 Variation in retention factor and resolution due to change temperature of modifier pump head

Conditions	k Nic	k Cot	k Ana	k Nor	Rs (Nic-Cot)	Rs (Cot-Ana)	Rs (Ana-Nor)
pump head at 19°C	0.206	0.651	1.077	2.702	2.439	1.839	4.552
pump head at 24°C	0.209	0.656	1.091	2.726	2.461	1.881	4.596
pump head at 27°C	0.207	0.658	1.092	2.764	2.474	1.871	4.686
pump head at 34°C	0.205	0.659	1.090	2.775	2.507	1.875	4.759
% change (19°C-34°C)	0.5	1.2	1.2	2.7	2.8	2.0	4.5

Conditions: Diol column, 0.5ml/min, 50°C, 8% MeOH + 1.025% iso-PA, 200kg/cm^2.

The changes were minor, however the influence of the laboratory temperature on the modifier should be considered, when separation are performed, in which the retention of the compounds is very susceptible to changes in modifier concentration.

In summary, the following parameters influence the reproducibility of the separation of the alkaloids: water content in the stationary phase, water content in the mobile phase due contaminated modifiers and additives or absorption of water from the atmosphere during pumping, pump head temperature of CO_2 and modifier pump and the temperature of the CO_2 cylinder.

3.1.4 Separation of Alkaloids on (S)-NEC-β-CD Column

Before beginning this study, the area reproducibility of the individual peaks was tested at different temperatures with the injector mounted either inside or outside the oven. This was important so as to ensure reproducibility while investigating the influence of temperature.

The original set-up of the instrument as described in 2.2.2 had the injection valve installed inside the oven. The addition of a polar additive was necessary as nicotine eluted as a broad peak (k= 1.06) when only methanol was used as modifier. The addition of 2.5% water to methanol resulted in a broad, fronting peak (k= 4.9),

however when diethylamine at a concentration of 0.5% v/v was added, nicotine eluted as a sharp and non-tailing peak (k= 0.589).

The use of basic additives were not included in the calculations of the critical parameters as no published values were readily available for comparison and the calculations for ternary mixtures would be very cumbersome. As the calculations only provided a rough guide, it was, assumed that the very small additions of aliphatic amines did not influence the critical parameters severely.

The RSD of 5 nicotine injections in a temperature range from 30 - 70°C was investigated and the results can be seen in Table 3.11. The conditions listed in Table 3.4 were used for this study because it provided a rapid analysis of the alkaloids.

Table 3.11 Retention time and area reproducibility while injector was mounted internally

Oven Temperature [°C]	Retention time reproducibility RSD [%]	Area reproducibility RSD [%]
30	0.16	0.72
40	0.18	0.85
50	0.20	0.97
60	0.21	1.08
70	0.24	14.55

SFC conditions: (S)-NEC-β-CD column, 1.0ml/min total flow rate, 15% MeOH containing 0.5% DEA, 200kg/cm^2.

The reproducibility of the area deteriorated at temperatures above 60°C due to the evaporation of MeOH in the injection valve. This relates to the boiling point of methanol at 65°C (Baker HPLC Solvent Manual). Greibrokk et al. (1989) indicated that the injector should be installed outside the oven when reproducible results using volatile solvents were desired. Table 3.12 gives the results obtained for the injection reproducibility for the injection valve mounted externally and shows clearly the improved area reproducibility even at higher temperatures. The connecting tubing had to be marginally longer for this set-up, however this should

not negatively influence the efficiency. Moreover, the results obtained correspond favourably with the injection reproducibility obtained by Pacholec et al. (1988) which were 0.3-1.18% RSD for retention times and 2.62-3.39% for area reproducibility. The RSD of the retention times in Table 3.12 was slightly higher than in Table 3.11, however this was a result of the manual start of the integrator when injection was initiated.

Table 3.12 Retention time and area reproducibility while injector is mounted on the outside

Oven Temperature [°C]	Retention time reproducibility RSD [%]	Area reproducibility RSD [%]
30	0.10	0.76
40	0.31	0.43
50	0.25	0.83
60	0.22	1.25
70	0.32	1.33

Conditions: as in Table 3.11, except injector was mounted externally.

Day to day reproducibility: Conditions in Table 3.11 were used to investigate the influence of temperature on retention over an oven temperature range of 30-60°C. An alkaloid mixture as described in 2.2.3 was used and triplicate injections were made. The same separation was conducted the following day to test whether there was good day-to-day reproducibility. Table 3.13 shows the findings, the results obtained the day after are given in brackets and these show distinct changes in retention times, particularly for anabasine. The retention time of methanol was used as dead time marker, even though Heaton et al. (1994a) and Berger (1995) observed that methanol was retained on columns and hence did not give an accurate dead time. In this study however, the calculated retention factors serve more as a comparison to investigate trends of given parameters on retention than absolute values from which physico-chemical constants would be derived. The requirement for very accurate dead times would further only be useful, if the

temperature of the pump head and the head pressure in the CO_2 cylinder were accurately known, as these influence the relative retention times significantly as discussed in 3.1.3.

Table 3.13 Investigation of the influence of temperature on retention and resolution.

Temperature [°C]	Chrom. Parameter	Nicotine	Cotinine	Nornicotine	Anabasine
30	k	1.79 (1.32)	2.14 (1.76)	5.46 (3.89)	10.99 (7.0)
	Rs	1.33 (2.10)	7.40 (6.26)	6.73 (5.69)	
	α	1.20 (1.33)	2.55 (2.21)	2.01 (1.80)	
40	k	1.42 (1.09)	2.15 (1.78)	5.70 (4.08)	8.71 (5.82)
	Rs	3.34 (3.51)	8.97 (6.84)	4.74 (3.53)	
	α	1.52 (1.64)	2.65 (2.29)	1.53 (1.43)	
50	k	1.23 (0.96)	2.29 (1.87)	6.16 (4.36)	6.80 (4.36)
	Rs	5.25 (4.96)	9.55 (6.49)	1.09 (0.58)	
	α	1.87 (1.94)	2.70 (2.53)	1.10 (1.07)	
60	k	1.09 (0.88)	2.37 (1.97)	6.35 (4.59)	5.07 (3.61)
	Rs	6.57 (6.20)	9.88 (7.19)	2.54 (2.29)	
	α	2.22 (2.24)	2.68 (2.34)	1.25 (1.27)	

SFC conditions: (S)-NEC-β-CD column, 1.0ml/min, 15% MeOH containing 0.5% DEA, 200kg/cm^2.

The values in the brackets show the results obtained after the column was heated to 60°C and a retention time reduction of 17-36% was observed with the greatest reduction experienced in anabasine retention (36%).

There may be various reasons for these marked changes. Firstly, the (S)-NEC derivatised stationary phase could be susceptible to the addition of the strong basic additive. Berger and Deye (1991f) however did not report stationary phase degradation when aminopropyl, octadecyl, and diol derivatised silica stationary phases were used in conjunction with strong amine additives. These phases are based on a silica matrix, thus are comparable to the chiral columns, in which the CD moiety is attached to the silica surface. Secondly, since initial trials were conducted using water as additive it may be possible there were still traces of water

present which caused the retention times to decrease slowly due to the slow removal of water. Water has a higher solubility in CO_2 at higher temperatures (Coan and King 1971), therefore when the separation at higher temperatures was conducted a higher proportion of water was removed from the stationary phase and this would account for observed decrease in retention factor when the analysis was repeated at every temperature (results in brackets). Ashraf-Khorassani et al. (1988a) observed a reduction in retention of basic compounds when a conventional cyanopropyl column using pure CO_2 was heated to 160°C, however no explanation was given.

The problems of irreproducible retention times were discussed in 3.1.3, however the phenomena was not due to degradation of the stationary phase, but due to the presence of water. The column was therefore conditioned overnight with a ternary mixture of CO_2, MeOH and DEA at 80°C to remove all water and allow the investigation of different parameters on retention.

Amine type and level: The following conditions were used for subsequent investigations unless stated otherwise: 0.5ml/min total flow rate, 15% MeOH containing 72mM amine, 60°C, 200kg/cm^2, back pressure regulator temperature 50°C, UV detection at 254nm.

Different types of amines and their corresponding pK_a as listed in Table 3.14 were used to investigate the influence of primary, secondary and tertiary amines and amines with different chain length. The concentration of the amines in MeOH were calculated in order to produce equal molar quantities.

The results are graphically represented in Figures 3.5 to 3.7 and show the influence of the amine nature retention factor k, resolution Rs and selectivity α, respectively. As seen from Figure 3.5 the nature of the amine did not change the retention factor markedly, although the more basic amines DEA and TEA caused a faster elution of nornicotine.

Table 3.14 Amine types, their pK_a[a] and their concentration in MeOH

Amine nature	Concentration [%]	pK_a
Triethylamine TEA	1.01	10.82
Diethylamine DEA	0.75	10.98
n-Octylamine OCA	1.60	10.65
n-Hexylamine HXA	1.27	10.64
n-Butylamine BTA	0.71	10.61
iso-Propylamine iso-PA	0.62	10.60
n-Propylamine NPA	0.59	10.69

[a] Perrin (1972, 1965).

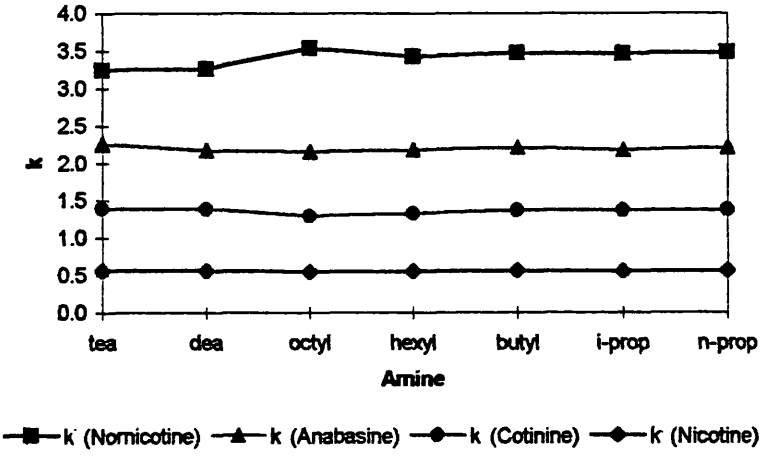


Figure 3.5 retention factor of alkaloids in dependence of amine nature.
Conditions: (S)-NEC-β-CD) column, 0.5 ml/min total flow, 60°C, 15 % MeOH containing 72mM amine, 200 kg/cm^2.

In contrast to the expected results that the stronger bases TEA and DEA would also give the most efficient separation, the resolutions as shown in Figure 3.6 using these two amines were significantly lower due to the poorer peak shape obtained with these amines. According to Berger and Deye (1991c, 1991d, 1991f, 1992) the main effects of additives on retention were by covering the residual silanols and ion suppression. The importance of steric hindrance was also highlighted and it

was found that strong basic amines could be eluted easier if they were sterically hindered (Berger and Deye 1991f).

The same appeared to be true for TEA and DEA. Despite being the stronger bases they are not able to deactivate the stationary phase or suppress ionisation of the alkaloids as efficiently as primary amines due to steric hindarance. The resolution of nicotine and cotinine was influenced to a far lesser extent than the resolution of anabasine and nornicotine, which was expected as nicotine and cotinine are the weaker amines.

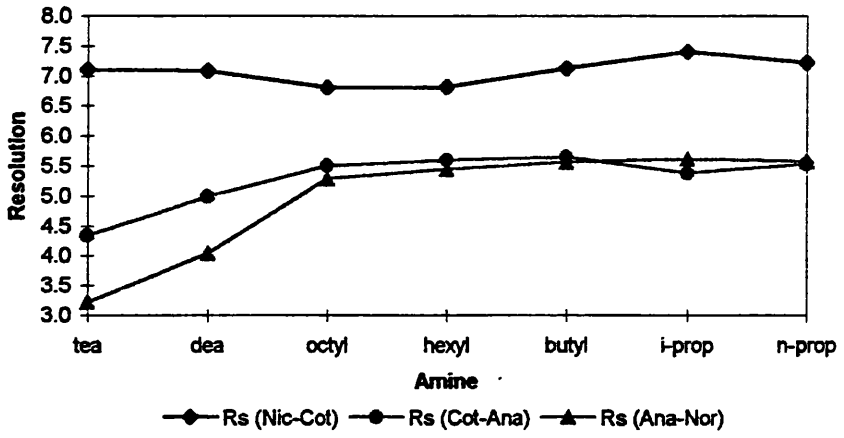


Figure 3.6 Resolution of alkaloid dependent on amine nature.
Conditions as in Figure 3.5.

Efficiency calculations using each alkaloid peak revealed that the early eluting nicotine peak had a substantially higher plate height (70μm) than cotinine (43μm) and anabasine (47μm) due to the dominant effect of extra-column band broadening for early eluting peaks. Nornicotine (63μm) also had a higher plate height due to the likely presence of a second retention mechanism, namely the interaction with residual silanol groups or due to insufficient ion suppression. High plate heights due to a mixed retention mechanism were also expected for anabasine as its pK_a is 11 and thus comparable to that for nornicotine. The reason for the narrow peak width for anabasine was due to peak suppression which occurs frequently in ion-

pair chromatography as discussed by Fornstedt and Westerlund (1993). Therefore only the plate height calculated from the cotinine peak should be used for efficiency evaluation. Resolution was calculated as in Experimental 2.2.5, however it was observed that the measured base peak width using the tangent method underestimated the actual peak width by about 30% and thus resulted in better resolution by a factor of 1.3 compared to results when tangents closer to the baseline (visual evaluation) were used. Bidlingmeyer and Waren (1984) recommended the use of the 5σ-method for the accurate determination of column efficiency. A good correlation with about 4% deviation between the visual determination and 5σ-method was obtained. Although the tangent method overestimated the resolution, it was preferred to the 5σ-method, which would be laborious, since the resolution values were only used for comparison between different operating conditions.

Selectivity was not significantly influenced by the nature of additive present in the mobile phase which can be deduced from Figure 3.7 and from the almost constant distances between the retention factors in Figure 3.5. Constant selectivity when changing the amine nature was also observed by Siret et al. (1992) for the separation of chiral compounds.

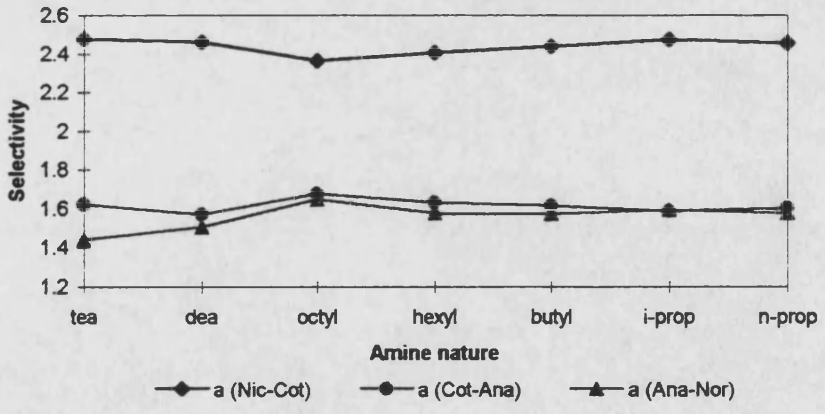


Figure 3.7 Selectivity of the alkaloids in dependence of the amine type.
Conditions as in Figure 3.5.

Selectivity is normally expressed with the symbol α, however this was not possible with the software used, so that α was substituted by a.

As seen from Figure 3.8 depicting the separation of the alkaloids using TEA, DEA and for comparison iso-PA, the primary amine was substantially more effective in controlling the retention mechanism.

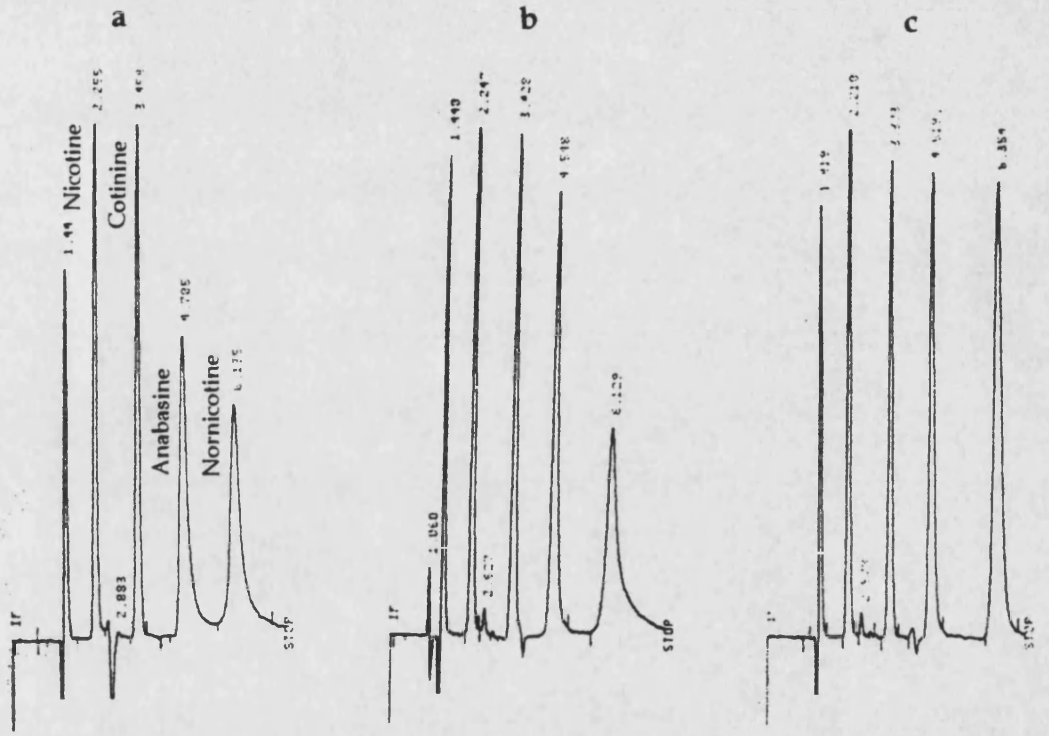


Figure 3.8 Separation of alkaloids using different amines a) TEA b) DEA c) iso-PA.

Table 3.15 Plate heights [μm] obtained for the alkaloid peaks using different amines as additives

Amine nature	Plate height of Nicotine peak [μm]	Plate height of Cotinine peak [μm]	Plate height of Anabasine peak [μm]	Plate height of Nornicotine peak [μm]
TEA	74	44	109	104
DEA	71	44	56	107
OCA	71	44	56	69
HXA	71	44	49	57
BTA	69	42	47	61
iso-PA	60	43	48	63
NPA	66	43	47	62

If the plate height was calculated using the nicotine or cotinine peak there was no significant improvement in using sterically less hindered or stronger amines, however there was a clear difference for anabasine and nornicotine. TEA was the least efficient with 100μm, achieving only 10% of the theoretical efficiency ($h_{min}= 2d_p$). However, the plate height given by the manufacturer is 44μm at a retention time of 5 minutes, thus 100μm corresponds to 44% of the expected efficiency. DEA achieved a plate height for anabasine comparable to the most efficient amines, but was unable to improve the plate height of the nornicotine peak. Octylamine improved the plate height of the anabasine and nornicotine peak in comparison to TEA and DEA, however did not achieve the low plate heights achieved by hexylamine, butylamine, iso-PA and n-propylamine. The plate height achieved for cotinine, whose evaluation was the most accurate one, showed that the same efficiency as given by the manufacture was achieved. The theoretical efficiency could not be achieved due to the less efficient packing of narrow bore columns.

Amine concentration: iso-PA was used for the investigation of the level of amines necessary to obtain the best separation, however hexylamine, butylamine and n-propylamine could equally have been chosen. As seen from Figure 3.9 the

resolution of anabasine and nornicotine increased from 0.4% up to an amine concentration of 1.23% in MeOH and appeared to then level off.

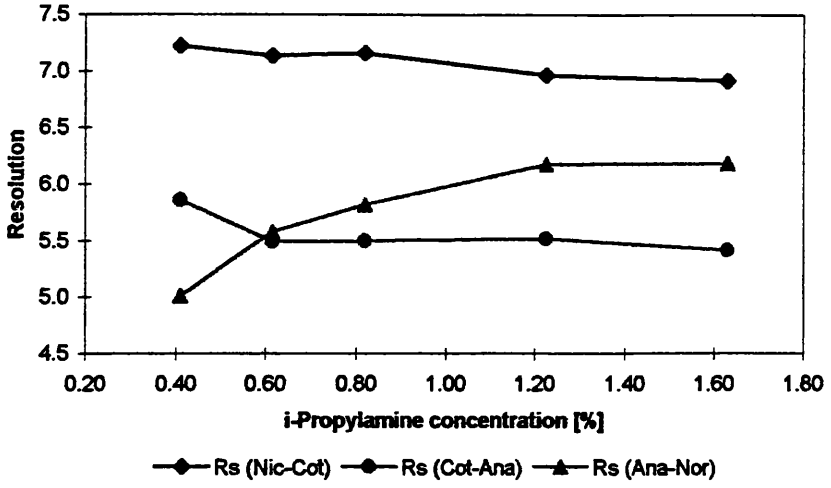


Figure 3.9 Dependence of alkaloid resolution on amine concentration.
Conditions as in Figure 3.5.

This was due to the suppression of the interaction of the alkaloids with silanols or ion suppression, producing a more defined separation mechanism and thus increasing efficiency. Further increase in amine did not influence the retention factor markedly as seen in Figure 3.10, suggesting that the primary function of the amine is not to increase solubility, but ion suppression or stationary phase deactivation and/or modification.

Berger (1995) interpreted the action of additives as either saturation of active sites, ion suppression or as a change in the stationary phase polarity. Deye et al. (1990) did not measure the solvent strength change when adding basic additives since Nile Red could not be used for a basic fluid mixture. However the authors noted that addition of a strong acidic additive to MeOH did not change the solvent strength markedly when less than 5% additive is added, confirming that the addition of amine does not influence solubility markedly. An additive concentration of 1.23% was chosen for the remaining experiments.

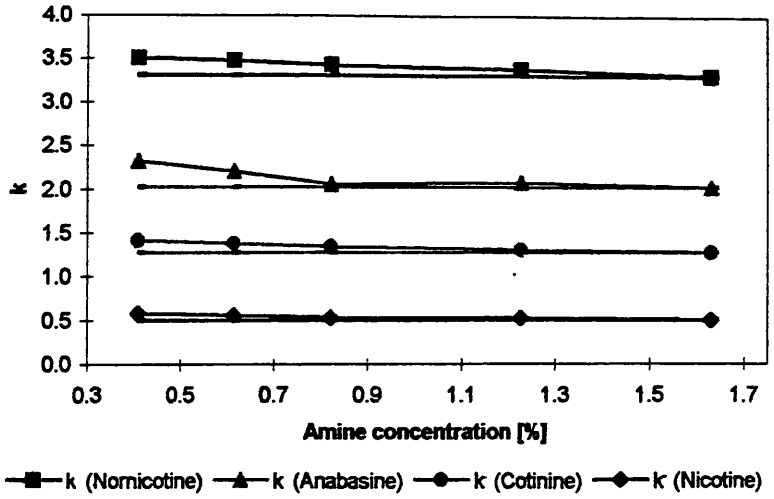


Figure 3.10 Variation of retention factor with increasing amine concentration.
Conditions as in Figure 3.5, straight line underneath.
each line indicates the final retention factor.

Influence of temperature: The most distinctive changes in chromatographic parameters were achieved by varying the temperature. As seen from Figure 3.11 the retention factor of anabasine was decreasing up to 90°C, due the dominant influence of vapour pressure increase on solubility, counteracting the loss in solubility due to a decrease in density. Cotinine and anabasine co-elute at around 80°C and thereafter a peak reversal was observed.

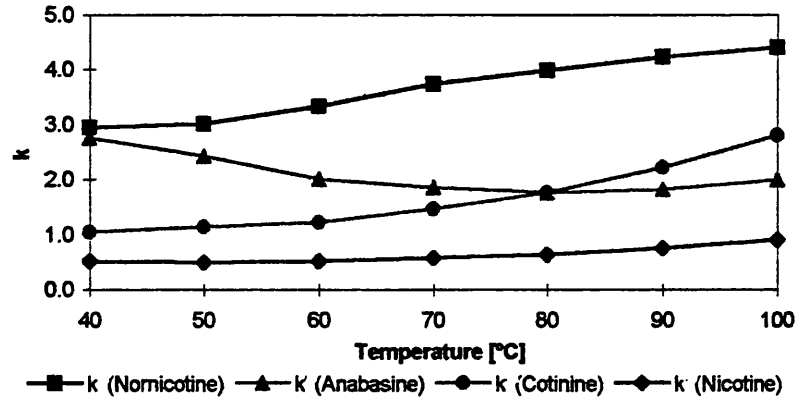


Figure 3.11 Influence of temperature on alkaloid retention.
Crossing lines indicate peak reversal.
Conditions: 0.5 ml/min, 15 % MeOH containing 1.23% iso-PA, 200 kg/cm^2.

At 100°C, however, the retention increases again, as the decrease in density was not balanced by a sufficient increase in volatility. As the retention times of nicotine, cotinine and nornicotine increase, the increase in volatility was insufficient to overcome the loss in density. Berger (1995) considered the argument of competition between volatility increase and solubility decrease with increasing temperature to be illogical, since only one phase is present. The reduction in retention times with increasing temperature was explained by the desorption of mobile phase components from the stationary phase, which decreased the stationary phase volume, hence causing a decrease in retention. It is, however, surprising that the behaviour of anabasine does not corresponds to the retention behaviour of nornicotine, despite of having almost identical boiling points. This may have been caused by an extreme temperature dependence of the anabasine and stationary phase interaction. Berger and Wilson (1994a, b) also observed the retention factor decrease and increase to varying degrees (Berger and Wilson 1994a, b) or to remain constant in various separations (Berger and Wilson 1995).

Due to the different retention behaviour of carbamate pesticides (Berger et al. 1994) the selectivity changed markedly with temperature. The same result was observed with the alkaloids and the influence of temperature on resolution and selectivity can be seen in Figure 3.12 .

The optimum temperature appeared to be 60°C as a compromise between the decreasing resolution and selectivity of cotinine-anabasine and increasing resolution and selectivity of nicotine-cotinine and anabasine-nornicotine. Additionally, efficiency was increasing with a concomitant increase in temperature, due to the increased diffusion coefficient, therefore the highest possible temperature should be chosen.

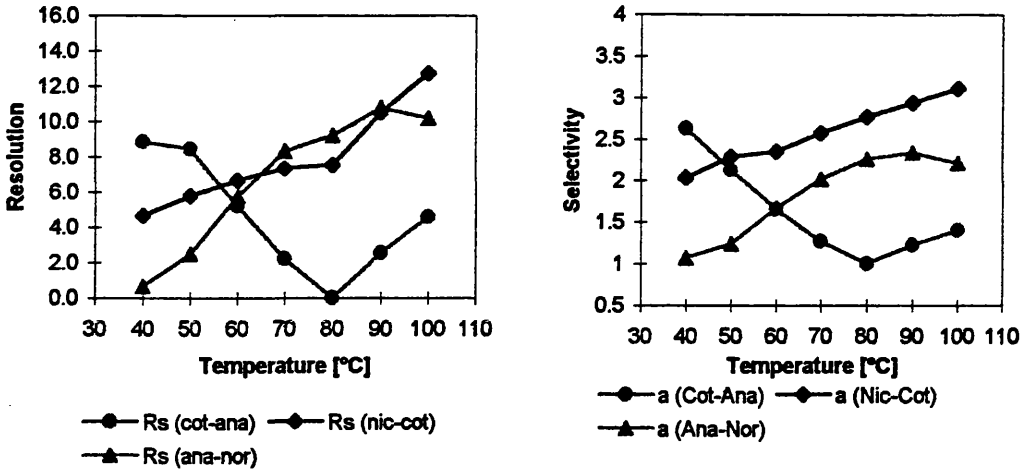


Figure 3.12 Influence of temperature on resolution and selectivity.
Conditions as in Figure 3.11.

Pressure: Since a pressure increase causes the density of the mobile phase to increase a decrease in retention was expected (Lee and Markides 1990). Figure 3.13 shows the decrease in retention factor with pressure and indicated clearly that the retention of nornicotine was most dependent on solubility. This was unexpected as nornicotine has almost the same boiling point as anabasine, however nornicotine has slightly higher pK_a value (pyrrolidine pK_a= 11.27 and piperidine pK_a= 11.22) and might be sterically less hindered, which might have caused stronger interaction with the stationary phase.

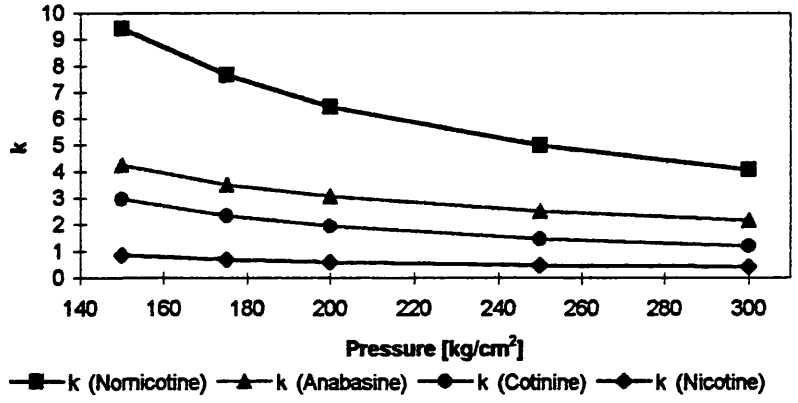


Figure 3.13 Pressure dependence of alkaloid retention.
Conditions as in Fig. 3.11, 60°C.

Since the retention factor of nornicotine was decreasing faster than that of anabasine the resolution and to a lesser extent selectivity between the two were also decreasing with increasing pressure. The retention of nicotine was only marginally affected by the pressure, thus the resolution and selectivity of nicotine-cotinine was decreasing with pressure due the decreasing retention time of cotinine. Berger and Wilson (1993b) also observed only a small influence of pressure on the retention of weakly basic anilines.

It is worth noting, t_0 measured with a MeOH injection increased with increasing pressure. The increasing t_0 could be caused either by the decreased adsorption of mobile phase onto the stationary phase as observed by Strubinger et al. (1991b) or due to the increased density. This caused the linear velocity to decrease, when a system comprising two reciprocating pumps running in a constant flow rate mode was used. The latter explanation certainly affected t_0, however the degree to which the two effects influenced t_0 is unknown. The same holds true for the temperature investigation since the increased temperature caused the density to decrease and the linear velocity to increase.

Modifier concentration: Increasing the modifier concentration decreased the retention factor due to the increasing solvent strength (Deye et al. 1990) of the mobile phase as seen in Figure 3.14. It was interesting to note the different extent to which the retention factors changed with increasing modifier concentration. This not only caused large changes in retention but also caused selectivity to change significantly.

An increase in modifier concentration increased the solvent strength of the mobile phase to a larger extent than a pressure increase, therefore the effects of modifier composition on retention, resolution and selectivity were the same as with pressure, although the degree of change was more pronounced. In contrast, Berger (1995) noted that changing modifier concentration was less effective in influencing selectivity than changing pressure.

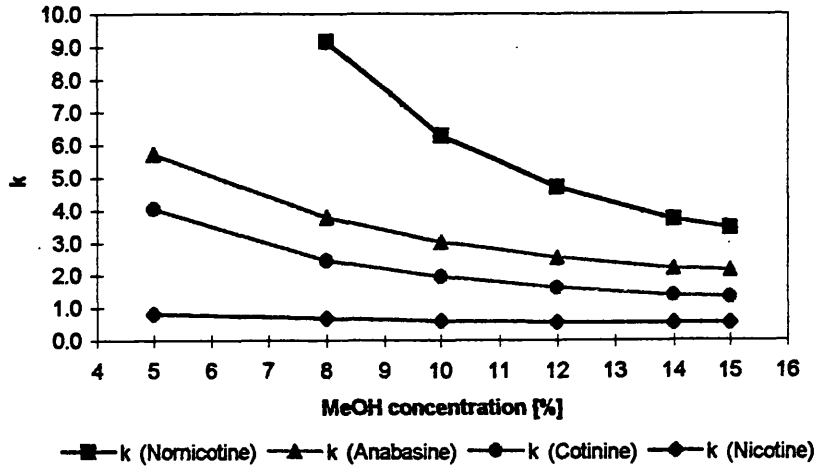


Figure 3.14 Modifier influence on the retention of alkaloids.
Conditions as in 3.6, 60°C.

Further indication that nornicotine was more difficult to elute from the column was suggested from the squared peak top up to 8% and slightly distorted peak up to 12% modifier concentration. Decreasing plate height for the nornicotine peak with increasing modifier is shown in Table 3.16, despite the increasing density of the mobile phase due to the increase in solubility. In contrast, the efficiency of the cotinine peak changed markedly when the modifier concentration was increased from 5 to 8% due to the decrease in diffusion coefficient, when higher modifier concentrations are used.

Table 3.16 Plate height evaluation using the cotinine and nornicotine peak at different modifier concentration

Modifier concentration [%]	Plate height (Cotinine) [µm]	Plate height (Nornicotine) [µm]
5	26	-
8	40	88
10	44	78
12	49	73
15	45	63

Flow rate: To investigate the influence of flow rates, 10% modifier was used as higher flow rates than 0.5ml/min were applied. Otherwise, retention times would decrease to low values, causing extra-column band broadening to influence the peak width as observed for the early eluting nicotine peak. The solubility of nornicotine was quite low and square topped peaks were obtained, thus the plate heights of nornicotine were not considered. Increasing the flow rate resulted in an almost constant retention factor and hence selectivity. The same result was reported by Kot et al. (1994) for the separation of basic and acidic drugs on chiral columns. The authors observed a plate height of 25μm for propanolol using 30% MeOH containing 0.5% DEA. The reason for the higher plate heights in the separation of the alkaloids using a (S)-NEC-β-CD column could be the inherent lower efficiency of cyclodextrin columns (Jefferies 1995), the lower efficiency of smaller i. d. columns or the dominance of extra-column band broadening. All connecting tubing was however kept to a minimum length and a small diameter tubing of 0.005" i. d. was used and the 8μl UV detection cell should not have caused excessive extra-column band broadening. Figure 3.15 show resolution and selectivity respectively.

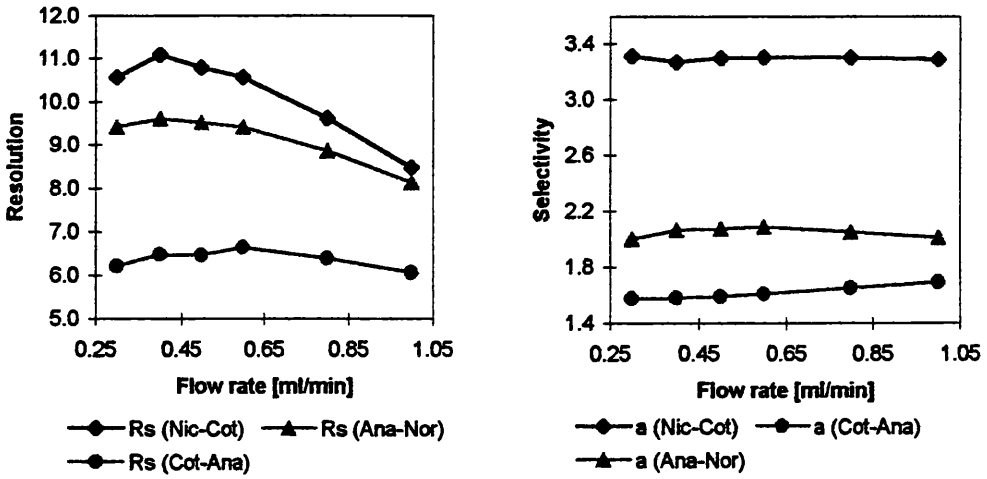


Figure 3.15 Influence of flow rate on resolution and selectivity.
Conditions: 60°C ,10% MeOH containing 1.23% iso-PA, 200kg/cm^2.

Under these conditions a flow rate of 1.0ml/min can be used as the resolution and selectivity were large enough to obtain a good separation.

Calibration: Calibration standards were prepared by diluting the stock solutions to the appropriate concentrations and analysing the solutions using the following conditions: 60°C, 10% MeOH containing 1.23% iso-PA, 200 kg/cm^2, 0.5 ml/min total flow rate. As seen from the calibration graph and the linear regression in Figure 3.16, the separation was linear over a wide range of concentrations, even for nornicotine which did not have an absolute symmetrical peak. At 5.1ng injected nicotine the signal-to-noise ratio is 10, therefore approximately 1.5ng could be detected at a signal-to-noise of 3, however the quantification would be most likely less accurate. Nornicotine could not be quantified at these low concentrations due to the noisy baseline and the broader peak obtained at longer retention times.

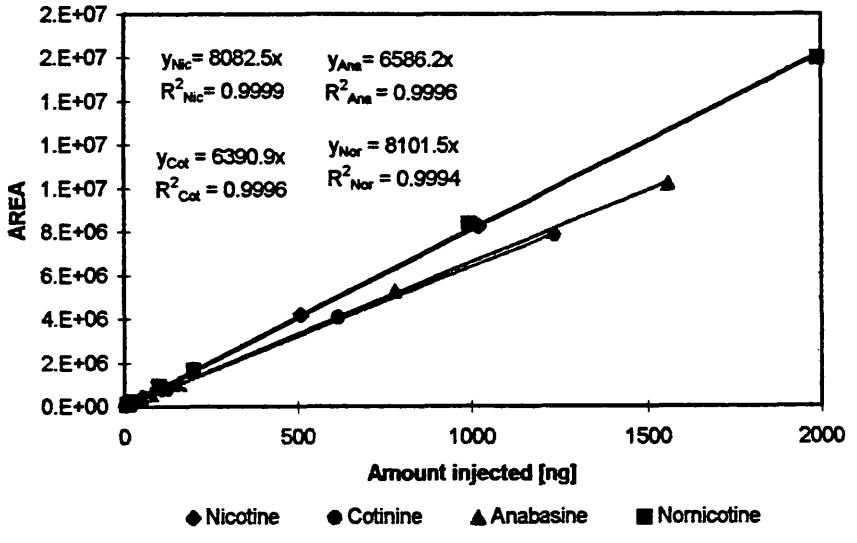


Figure 3.16 Calibration curve of the alkaloids.
Conditions: 0.5ml/min flow rate, 60°C, 10% MeOH containing 1.23% iso-PA, 200kg/cm^2.

The results are comparable with the calibration results of Saunders et al. (1981), who found a linearity between 50ng and 7000ng. However a better correlation coefficient was obtained than that cited by Janicot et al. (1988) for the quantification of opium alkaloids. Correlation coefficients between 0.9941 and 0.9986 were found

in the study of Janicot et al. (1988), thus the correlation coefficient given in Figure 3.16 compared favourably.

3.1.4 Separation of Alkaloids on β-CD column

An initial trial to elute the alkaloids with 10% MeOH at 50°C, 1.0ml/min and 200kg/cm^2 resulted in a slightly tailing peak for nicotine at k= 0.66 and a non-tailing peak for cotinine at k= 1.26. Nornicotine and anabasine eluted as very broad "peaks" at around 10 minutes, therefore the addition of strong amine additives was necessary. The experiments were carried out using 1.0ml/min as this enabled quicker equilibration and allowed additional experiments to be conducted.

When MeOH was substituted as modifier with 20% MeCN or 25% 2-PrOH containing 0.5% DEA, better separation of tobacco alkaloids on β-CD was expected, since Armstrong et al. (1990) reported superior separation efficiency when using MeCN as the organic solvent in RP-HPLC. However, when MeOH was replaced by MeCN or 2-PrOH in SFC the resulting chromatograms showed broad peaks for anabasine and extremely broad peaks for nornicotine with substantially longer retention times. This indicated the lower solvent strength of the fluid mixture when either MeCN or 2-PrOH was used as a modifier and further confirmed the lower solubility of nornicotine compared to anabasine. The addition of amine should have suppressed ionisation to the same extent in all three modifiers, since however tailing peaks were observed for MeCN or 2-PrOH it proved that good solubility is essential to achieve symmetrical peaks. It further demonstrated that the amine addition does not influence solubility to a great extent. However, no conclusion could be drawn to what extent the good elution with MeOH was caused by either solubility or stationary phase effects.

Amine type and level : Using 15% MeOH with 72mM amine, TEA and DEA eluted nornictotine slightly earlier than with the longer chain primary amines.

These amines are likely to be more efficient in ion-suppression, however less able to deactivate the underlying silica surface due to steric hindrance. The resolution between nicotine and cotinine was lower on the β-cylcodextrin column than on the (S)-NEC-β-CD, since the N-substituted amines did not interact as strongly with the hydroxylgroups as anabasine and nornicotine on the β-CD ring and no additional π-π interactions were present as in the (S)-NEC-β-CD column. Figure 3.17 shows the resolution achieved with the different amines suggesting that DEA was more efficient in resolving the alkaloid pair anabasine-nornicotine on the β-CD column than on the (S)-NEC-β-CD column.

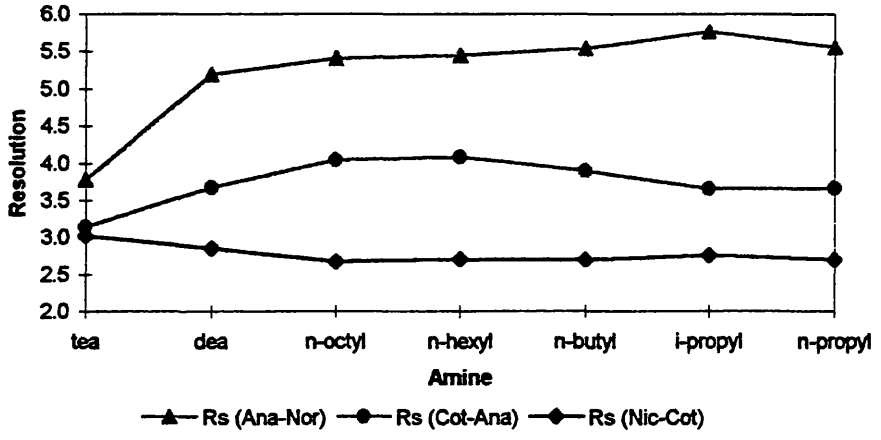


Figure 3.17 Resolution of the alkaloids dependent on the amine structure.
Conditions: β-CD column, 1.0 ml/min, 60°C, 15% MeOH containing 72mM amine, 200kg/cm^2.

All 7 amines were used to investigate optimum levels of amine in MeOH and the same trend could be observed with each of the amines. The retention factor of the alkaloids only slightly decreased and the resolution between anabasine and nornicotine improved to a maximum level and then levelled off. The optimum amine concentration for all amines investigated was at around 72mM in MeOH which was equivalent to 0.615% iso-propylamine in MeOH. This was considerably lower than the optimum level of 1.23% iso-PA in MeOH for the (S)-NEC-β-CD column, which might suggest that fewer silanols were present in the β-CD column or less adsorption of amines took place on the stationary phase.

Temperature : As retention of the alkaloids was considerably lower on the β-CD column the temperature investigation was conducted using 10% MeOH containing 0.925% iso-PA. The retention of nicotine, cotinine and nornicotine increased with temperature, with nicotine showing the weakest response. The retention factor of anabasine decreased up to 80°C and then increased up to 100°C, causing the anabasine and cotinine to co-elute at 80°C, the same temperature as on the (S)-NEC-β-CD column.

Pressure: Increasing pressure decreased the retention factors of all four alkaloids, however as the retention factor of nornicotine decreased faster than that of anabasine, it caused the resolution of nornicotine-anabasine to decrease as shown in Figure 3.18. The same trend was observed for cotinine and nicotine as the retention of cotinine decreased to a greater extent. The resolution of cotinine-anabasine remained almost constant as both alkaloids showed the same dependence on pressure.

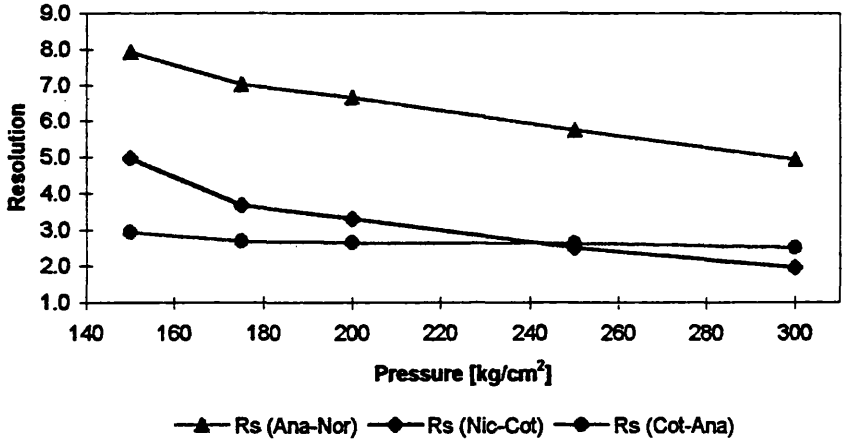


Figure 3.18 Pressure influence on the resolution of alkaloids.
Conditions: 1.0 ml/min, 60°C, 10% MeOH containing 0.925% i-propylamine, 200kg/cm².

Evaluation of the plate height indicated that higher plate heights were obtained with increasing pressure due to the decreasing diffusion coefficients of the alkaloids in the denser CO_2 fluid mixture.

Modifier concentration: The influence of the modifier was conducted at 40°C, 60°C and 100°C and the dependence of the retention factor with modifier concentration and temperature is shown in Figure 3.19. Increasing the modifier concentration in the fluid mixture increased the solvent strength of the fluid and hence caused the retention factors of the alkaloids to decrease.

The diagrams in Figure 3.19 highlight interesting trends in solubility. At a temperature of 40°C the retention factor of anabasine was strongly influenced by the MeOH concentration, whereas at 60°C the retention factor showed a decreased dependence on modifier. This was due to the already increased solubility of anabasine at 60°C or reduced interaction with the stationary phase as seen in the smaller retention factor compared to that at 40°C. The increased solvent strength of the mobile phase therefore had only a minor effect on the retention of anabasine.

When the retention of a compound decreases with increasing temperature it may have been caused by two factors as discussed on page 119. Moreover, the retention factor of anabasine at 100°C was higher, which indicated lower solubility, and hence the decreasing solvent strength of the mobile phase had a more pronounced effect. A similar trend was observed for the retention of cotinine, nicotine and nornicotine, since their solubility decreased with increasing temperature. The higher the temperature the more pronounced the effect of the concentration of MeOH on the retention factors. Furthermore, the higher the retention factor the greater the reduction in retention factor with increasing modifier concentration, hence resolution decreased with increasing MeOH concentration. The only exception to this trend was anabasine and cotinine at 60°C as both alkaloids experienced the same degree of retention reduction, causing the resolution to remain constant with increasing MeOH concentration.

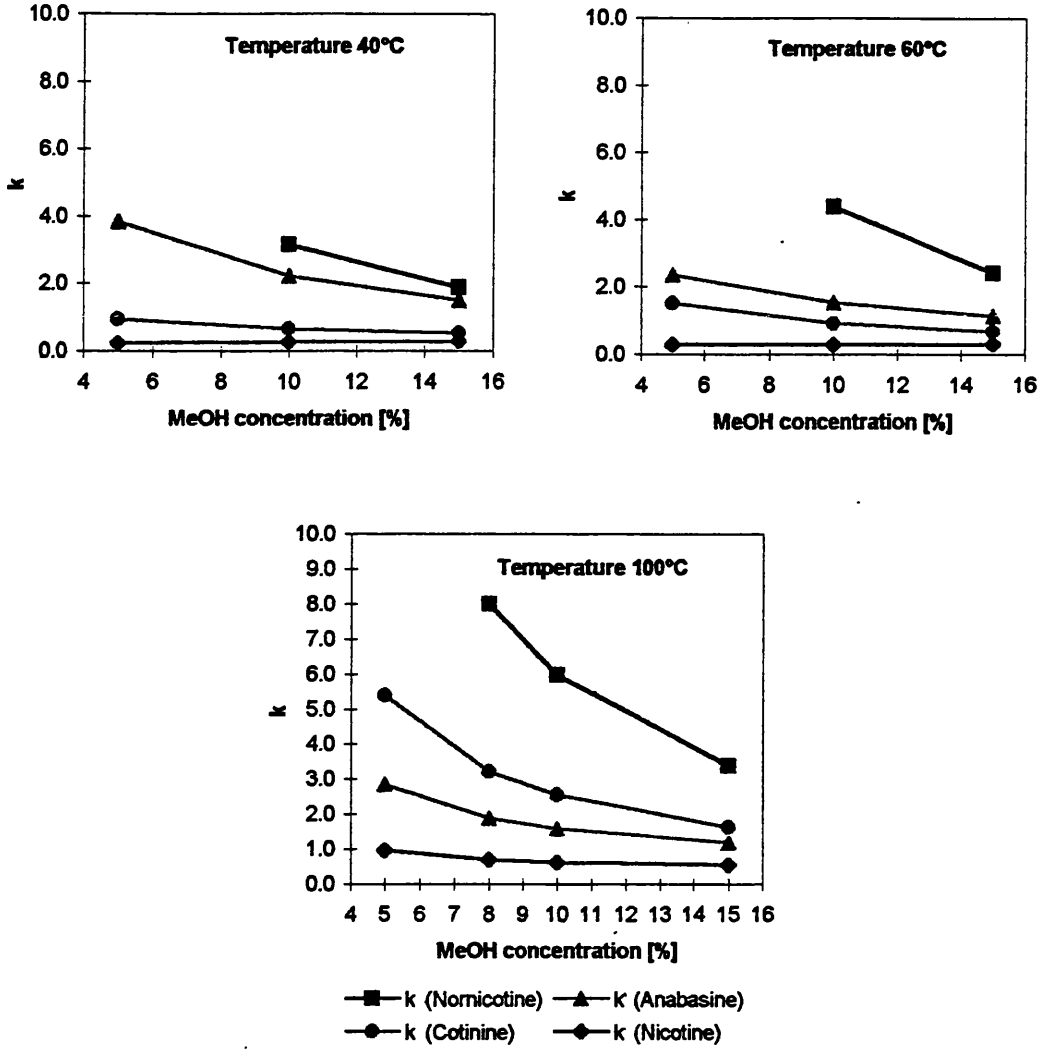


Figure 3.19 Influence of modifier on retention factor at three different temperatures. Conditions as in Figure 3.18.

Flow rate: The influence of flow rates on the separation of the alkaloids was investigated at two different temperatures in order to highlight the effect of temperatures with a concomitant increase in flow rate as depicted in Figure 3.20.

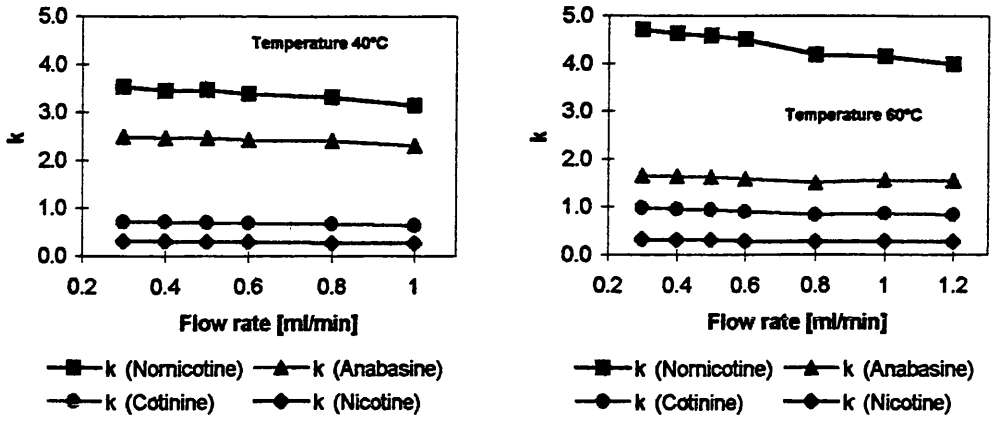


Figure 3.20 Influence of flow rate on retention factor at 40 and 60°C.
Conditions as in Figure 3.18.

As seen from the diagram at 60°C the retention factors of nornicotine appeared to decrease to a greater extent with increasing flow rate than at 40°C. This could have been due to the solubility limitation of nornicotine at these conditions, since increasing flow rate had the same effect as a fluid with increasing solvent strength. However, as the retention factors of all alkaloids decreased to an extent, the greater dependence of the retention factors on flow rate at the higher temperature might have been due to the additional increase in flow rate at the higher temperature because of the fluid's greater expansion. Since the reduction in the retention factors was of a similar degree for all the alkaloids, the selectivity remained almost unchanged. The resolution of the compounds was decreasing with increasing flow rate as the separation process became less efficient due to the increasing contribution of resistance to mass transfer in the mobile phase with increasing flow rate which can be seen from the measured plate heights of cotinine in Figure 3.21.

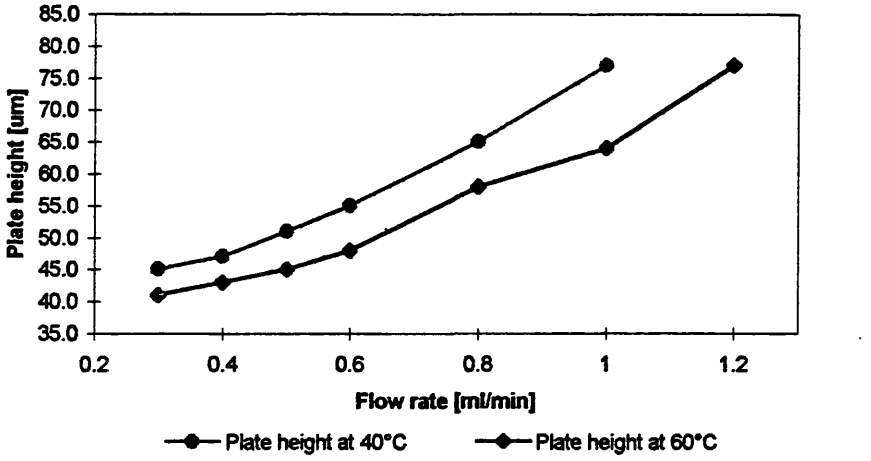


Figure 3.21 Plate heights of cotinine dependent on flow rate at 40 and 60°C. Conditions as in Figure 3.18.

The diagram depicts clearly that the separation was more efficient at 60°C, allowing the flow rate at higher temperatures to be increased to higher values and still be as efficient as lower temperatures.

3.1.5 Separation of Alkaloids on Diol Column

Initial attempts to elute the alkaloids with MeOH as modifier gave surprising results, in that as nicotine eluted only slightly tailed and very fast at k= 0.3 with 10% MeOH, 50°C and 200kg/cm^2. Cotinine eluted at k= 0.65 as a symmetrical peak and anabasine (1.78) and nornicotine (k= 2.9) also eluted at these conditions, although with severe tailing. The retention times were furthermore concentration dependent, which was another indication of the presence of a dual retention mechanism as observed by Schoenmakers et al. (1988). Since the alkaloids did not elute without tailing, the addition of a strong basic additive was necessary to suppress ionisation and interactions with silanol groups.

The investigation on the diol-column had to be conducted at 50°C since cotinine and anabasine almost co-eluted at 60°C and this would make evaluation difficult.

Furthermore, because the alkaloids were retained to a far lesser extent on the diol-column, only 8% MeOH was used.

Amine type and level: Only triethylamine, hexylamine, butylamine, iso-propylamine and n-propylamine were used to determine the most efficient amine, as the other amines in the series were contaminated with water or higher amines which were formed by polymerisation reaction (Jenkins 1995). The elution order of the alkaloids was the same as on the (S)-NEC-β-CD and β-CD columns and Figure 3.22 shows the effect of different amines on the resolution of the alkaloids. Hexylamine and butylamine achieved a markedly less efficient separation than the shorter chain amines, which was probably due to the steric hindrance in their adsorption onto the stationary phase. They were therefore less efficient in suppressing the interaction with the residual silanols. The resolution of the cotinine-anabasine decreased due to the more efficient and faster elution of anabasine with the shorter chain amines.

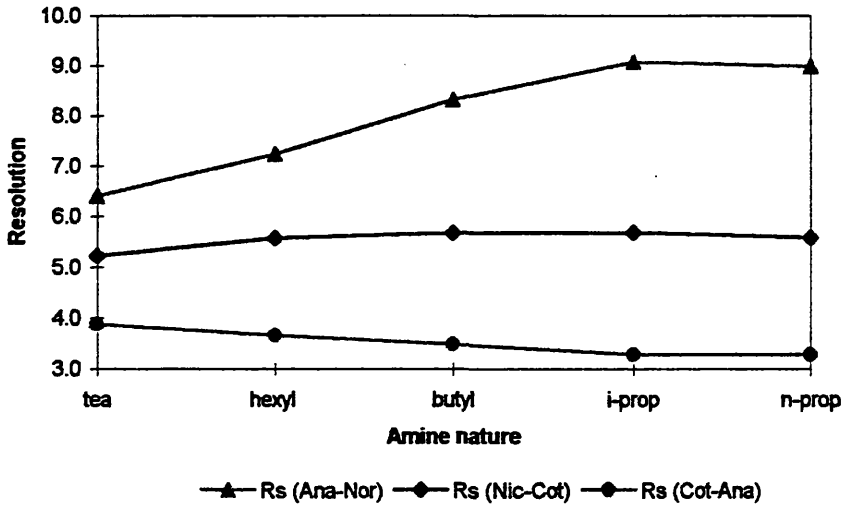


Figure 3.22 Resolution of the alkaloids dependent on amine type.
Conditions: Diol-column, 0.5 ml/min, 50°C, 8% MeOH + 64mM amine, 200 kg/cm^2.

This was not observed with the cyclodextrin columns, which suggested that for the adsorption of amine on the cyclodextrin cavity sterical hindrance is less important.

Iso-PA and n-propylamine showed the same efficiency in resolving the alkaloids hence iso-PA was chosen for a comparison with the (S)-NEC-β-CD and β-CD column. Since no definitive optimum was found for iso-PA in MeOH the same concentration as used in Figure 3.23 (1.025% iso-PA) was used for the following investigations.

Temperature: The retention factor of nornicotine increased up to 90°C and then decreased. Since solubility and volatility were the same in all three columns, the decrease in retention factor was due to the decreased interaction of nornicotine with the stationary phase. These were disrupted by higher temperature to a greater extent than on (S)-NEC and β-CD column. Unexpectedly, the retention factor of anabasine gave a minimum at 70°C with higher values at 40 and 100°C, indicating either stronger interaction of anabasine with the stationary phase or solubility limitations since a lower modifier concentration was used than on the other two columns.

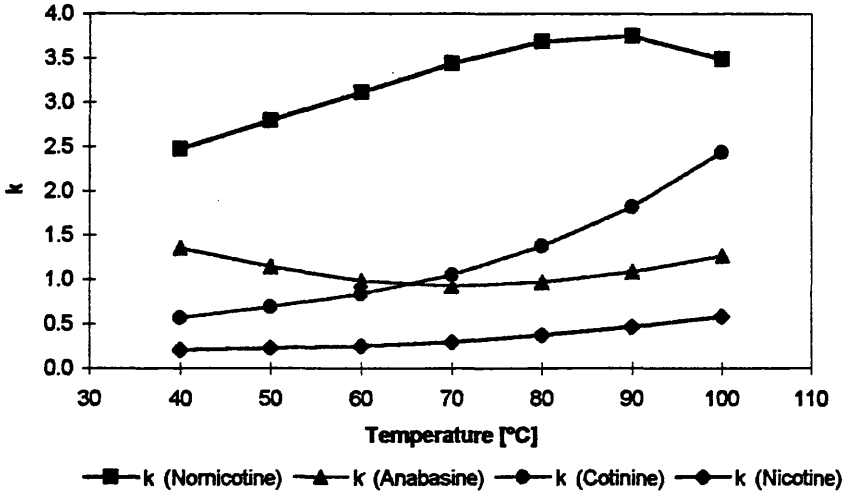


Figure 3.23 Retention factors dependent on temperature.
Conditions: Diol column, 0.5ml/min flow, 8% MeOH + 1.025% iso-PA, 200kg/cm^2.

As seen from Figure 3.23 the elution order of cotinine and anabasine reversed at a temperature of about 67°C. The Figure shows a further continuous increase in selectivity between the nicotine-cotinine pair, which can be deduced from the distance between the retention factors, until a minimum for cotinine-anabasine selectivity at about 67°C. A maximum for anabasine-nornicotine selectivity is observed at about 80°C. The resolution between the pairs showed the same trend as for the selectivity as seen in Figure 3.24.

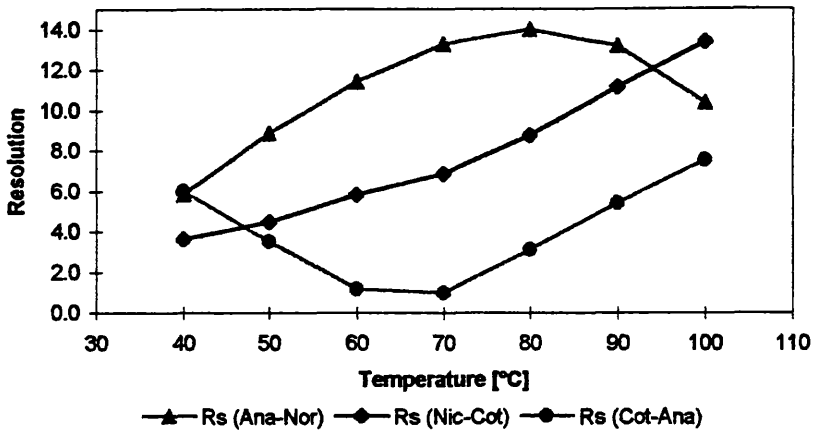


Figure 3.24 Relationship of alkaloid resolution with temperature.
Conditions as in Figure 3.23.

Presssure: An increase in pressure in the region of 150 - 300kg/cm^2 decreased the retention times of all four alkaloids and as seen before, the retention factor of nornicotine changed to the greatest extent, anabasine and cotinine to the same extent and nicotine changed only insignificantly with increasing pressure. This caused the resolution of anabasine-nornicotine and cotinine-nicotine to decrease and the resolution of anabasine-cotinine to remain constant. Selectivity of all three pairs did not change more than 0.25.

Modifier: Increasing the modifier concentration from 4-15% caused the retention factors to decrease, most markedly for nornicotine. Resolution decreased substantially with an increase in modifier for anabasine-nornicotine and nicotine-

cotinine, however only decreased slightly for cotinine-anabasine. Selectivity decreased for the former two pairs and increased slightly for contine-anabasine.

Flow rates: The retention factor decreased slightly during the flow rate increase, and again resolution decreased due to the less efficient separation at higher flow rates, which was also indicated by the increasing plate height with increasing flow rate. In contrast, selectivity remained constant over the flow rate increase of 0.3 - 1ml/min.

Calibration: The same freshly prepared standard injected onto the (S)-NEC-β-CD column were also analysed on the diol-column, although at a temperature of 80°C, to ascertain that the calibration would be linear at higher temperature. Good correlation coefficients were obtained as seen in Figure 3.25, proving that no irreversible absorption took place under the applied conditions.

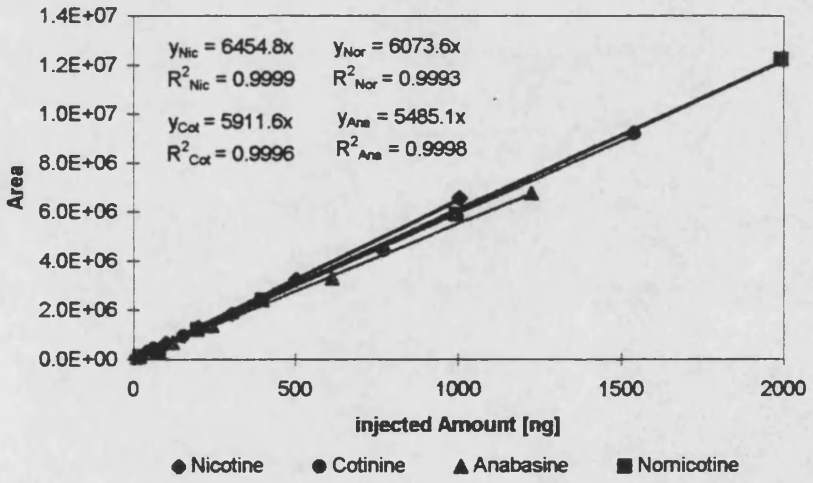


Figure 3.25 Calibration of alkaloids on a diol column.
Conditions: 0.5ml/min, 80°C, 7% MeOH + 1.025% iso-PA, 200kg/cm^2.

Berger and Deye (1991f) investigated the elution of amines and noted that it was impossible to elute benzylamine (pK_a= 10.86 Perrin 1981) without the addition of a strong basic additive. Whereas it was possible to elute nicotine and cotinine with more or less symmetrical peaks ($pK_a \sim 8$), there was no elution of anabasine and

nornicotine ($pK_a \sim 11.2$) on the two CD columns. However, it was possible to elute anabasine and nornicotine from the diol column, although with severe tailing. The addition of strong amines enabled Berger and Wilson (1993b) to elute benzylamine with high efficiency from diol columns (h= 2.27). Such low reduced plate heights could not be obtained in this investigation, the average reduced plate height was about 8, which was almost four times less efficient than that quoted by Berger and Wilson (1993b). The difference was most likely due to the use of small diameter columns in this investigation in contrast to the 4.6mm i. d. columns used by Berger and Wilson, in order to exclude any extra column effects.

3.1.6 Comparison of Alkaloid Separation on Different Columns

The separation of the four alkaloids was compared on a (S)-NEC-β-CD, β-CD and a diol column at the following conditions: 0.5ml/min flow rate, 50°C, 10% MeOH containing 1% iso-PA and 200kg/cm^2. The structure of a (S)-NEC-β-CD column is given in Figure 3.26. Additionally, the separation of the alkaloids was conducted on a silica column, to investigate the influence of silanol groups. However, the retention time of nornicotine continued to change by 1% within a day even after a overnight equilibration, where a retention time change of 20% for nicotine and cotinine occurred and 40% for anabasine and nornicotine. The values given in Table 3.17 may not represent constant retention times for the silica column.

Table 3.17 Comparison of the separation of alkaloids on a (S)-NEC-β-CD, β-CD, diol and a silica column

Column	t_0	RT (Nic)	RT (Cot)	RT (Ana)	RT (Nor)	k (Nic)	k (Cot)	k (Ana)	k (Nor)	α (Nic-Cot)	α (Cot-Ana)	α (Ana-Nor)
SNEC	1.561	2.465	4.359	7.672	11.348	0.579	1.792	3.915	6.270	3.095	2.184	1.602
Beta	1.639	2.179	3.067	5.026	8.163	0.330	0.872	2.067	3.982	2.642	2.371	1.926
Diol	1.683	2.142	2.832	3.802	5.932	0.273	0.683	1.259	2.525	2.502	1.844	2.005
Silica	1.518	2.350	3.156	5.227	5.227	0.548	1.079	2.443	2.443	1.968	2.265	1.000

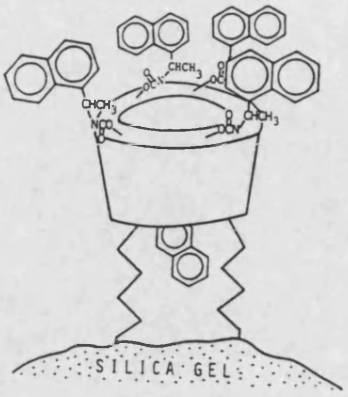


Figure 3.26 Structure of a (S)-NEC-β-CD column

As seen from the Table 3.17 retention times of the alkaloids on the (S)-NEC-β-CD column were considerably longer, confirming the presence of additional interactions, namely π-π interactions. Comparing the retention on the β-cyclodextrin column to the diol column, there must be additional interactions between anabasine, nornicotine and the β-cyclodextrin stationary phase and to a lesser extent for cotinine and nicotine. One explanation may be an inclusion complex or so called lid-rim interaction of the analyte and the cyclodextrin moiety. A more detailed discussion of inclusion or lid-rim complexes is given in section 4, however inclusion complexes appeared to be more plausible, as a lid-rim complex should have given similar retention to that on the diol column. Higher concentrations of free silanols, which could have been an alternative explanation, could not have been responsible for the increased retention of anabasine and nornicotine on the β-cyclodextrin column, since the retention of nornicotine was weaker on the silica column. It was observed that the presence of silanols did not contribute to the separation process, even for basic amines due to the distance of the cyclodextrin moiety from the surface and the high degree of coverage blocking

the access of small analytes to the surface (Technicol 1992). Moreover, the presence of a strong amine additive should have deactivated the silanols rendering these incapable of interacting with the analytes. However, Berger and Deye (1991c) noted that additives with higher acidic strength than the analytes were necessary to completely suppress ionisation. Since in this investigation iso-propylamine with a pK_a of about 10.6 (Perrin 1981) was used as basic additive and the analytes anabasine and nornicotine had a pK_a of about 11 (Perrin 1965), it could be that the incomplete ion suppression was responsible for the many equilibration problems encountered. Berger and Deye (1991c) concluded that for complete ion suppression the additive should have a pK_a of at least one unit lower for acids and found sufficient ion suppression for bases when additives with a similar pK_a were used (Berger and Deye 1991f). There was however no reference to day-to-day reproducibility of the retention or related problems. The strongest amine eluted in this reference was benzylamine with a pK_a of 10.6, however in the present investigation amines with a pK_a of over 11 were eluted. Diethylamine, which was also used, had a pK_a of 10.98 (Perrin 1981), however, was less efficient to produce narrow and symmetrical peaks for anabasine and nornicotine, demonstrating that steric effects were dominant over basicity. The same was observed by Berger and Deye (1991e) when analysing tertiary, secondary and primary amines.

In contrast, anabasine and nornicotine co-eluted on the silica column due to the dominance of the interaction of the secondary amine group with the silanol groups. It could therefore be concluded that the additional methylene group in anabasine did not influence the retention. On the diol and β-cyclodextrin column however, there were additional interactions present, causing anabasine and nornicotine to be resolved. On the diol column the hydroxyl groups are linked to the silica surface via a hydrocarbon linkage and analytes can therefore interact with both the hydroxyl groups and the hydrocarbon linkage, which seem to be responsible for the resolution of anabasine and nornicotine. As the retention of anabasine and nornicotine on the β- cyclodextrin column was greater than on the

diol column, the presence of a inclusion complex in the cyclodextrin moiety can be assumed.

3.1.7 Equilibration Times

Trials monitoring the absorption of amine at a wavelength of 210nm revealed the slow adsorption of amine onto the diol column, which explained the long equilibration time when the column was stored in MeOH overnight. It took at least 25min for the amine to break through, which included the dead volume of two mixing columns and the adsorption of amine. However, from the point of amine detection to a constant base line another 60 minutes passed when 8% MeOH containing 1.025% iso-PA was used for the equilibration. The complete elution of the amine with 8%MeOH without iso-PA appeared to be not possible, as the baseline did not return to the original level. Faster equilibration was possible when 20% MeOH containing 1.025% iso-PA was used, and complete elution of the amine was achieved when the column was rinsed with 20% MeOH. Column equilibration using 20% MeOH + 1.225% iso-PA took place in a total time of 50 minutes, 35 minutes faster than 8% MeOH containing the same amount of amine.

Further experiments were conducted to determine the equilibration times of the columns when changing physical parameters such as temperature, modifier, pressure and flow rate. The equilibration can be accelerated by using a higher flow rate of 1.0ml/min for 15 minutes and then equilibrating the column for 20 minutes at the desired flow rate, thus reducing the equilibration time by 45 minutes. The same effect could be achieved by using higher modifier concentrations, thus reducing the equilibration time.

Modifying the temperature of an equilibrated system required about 30 minutes for re-equilibration and 20 minutes for a change in pressure, modifier and flow rate

given that no water was present, as this prolonged the equilibration time considerably.

3.4 Separation of PCBs

The widespread use of PCB mixtures up to 1973 in closed and open systems, is responsible for the ubiquitous presence of PCBs in almost every component of the global ecosystem (Tanabe et al. 1987, Ericksen 1986). Over the years, concern has increased over the implications of the effect of PCBs on living organisms.

3.4.1 Introduction

Polychlorinated Biphenyls (PCBs) form a group of 209 possible congeners with different levels of chlorination, ranging from mono- to decachlorobiphenyls. The structure and the systematic numbering of the carbon atom is given in Figure 3.27, and the systematic formula can be expressed with the formula $C_{12}H_{10-x-y}Cl_{x+y}$.

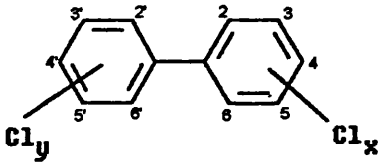


Figure 3.27 Structure of PCB's, indicating the numbering of the carbon atoms.

All 209 PCBs were numbered systematically by Ballschmiter and Zell (1980) according to a sequence that followed the IUPAC rules of substituent characterisation of biphenyls.

The use of PCB mixtures such as Aroclors and Clophen in closed and open systems were favoured for their exceptional physical and chemical properties, such as thermal stability, resistance to most chemicals, low flammability and high electrical resistivity (deVoogt 1989). Its use in open systems, e. g. in plasticisers, carbonless copy paper, lubricants, fire retardant etc. was the primary cause for the contamination of the environment with PCBs although to a lesser extent closed

systems such as transformers, capacitors, hydraulic fluid in mining equipment and heat-transfer systems have also caused environmental contamination. PCB mixtures used in these systems are produced by batch chlorination of biphenyl at temperatures above the melting point of biphenyl, but below 150°C (De Voogt 1989). Subsequent separation and purification results in PCB mixtures with a desired distribution of chlorinated PCB's.

The toxicity of the individual PCBs in these commercial mixtures varies greatly, because of their differences in molecular dimension and planarity (Bandiera et al. 1983 and Parkinson et al. 1983). The most toxic PCBs are characterised by the absence of ortho- substituted chlorines, which enables rotation around the central phenyl-phenyl bond. These PCBs are referred to as planar or coplanar and are capable of assuming the same configuration as the planar 2,3,7,8-tetrachlorodi-benzo-p-dioxin (TCDD). They are therefore able to display similar biochemical properties and toxicity as TCDD (De Voogt et al. 1990). PCB 77, 126 and 169 fulfil the structural requirements of being substituted in both para positions and at least one meta postition, therefore they are biologically the most active and most toxic compounds. Despite having the same structural characteristics, PCB 81 is less toxic than the above PCBs (De Voogt 1990). Moreover, similar mono-ortho and di-ortho substituted PCBs show comparable levels of biological activity to planar PCBs and therefore need to be included in the analysis. The determination of the total level of PCB's would not give a true reflection of toxicity as different PCBs have different biological activities. The best way to investigate the effect on wildlife and humans is by determining the individual PCBs and multiplying the resultant concentration with a so-called toxicity equivalent factor (TEF), which normalises the PCB concentration to the equivalent TCDD concentration, giving a true reflection of the level of contamination. The TEF was introduced by Sawyer and Safe (1982) and was based on the biological responses produced by the individual PCBs compared to TCDD. Since uptake, disposition and metabolism of the PCBs depend also on

the molecular structure and degree of substitution (Safe 1989), it is important to monitor the individual PCBs in order to assess bioaccumulation in the food chain.

De Voogt et al. (1990) discussed in an exceptional review of biological activity, the determination and occurrence of these planar, mono-and di-ortho PCBs and highlighted the need for improvement in the determination of planar congeners as most methods are too laborious and expensive.

One of the main problem originates from the low concentrations of the most toxic PCBs in commercial mixtures. As PCBs mixtures are normally present in small quantities in the environment, the most toxic PCBs are present at even lower concentrations, demanding highly selective detection methods. Erickson (1986) reviewed the determination of PCBs in a book, covering sample collection and storage, extraction, clean-up, determination and data evaluation.

Extraction of PCBs is normally achieved by liquid extraction, solid phase extraction, blending, ultrasonic extraction etc., which was reviewed by Hess et al. (1995). However, with the increasing popularity of SFE there have been numerous publications using this technique (Sweetman and Watts 1995, van der Velde et al. 1992, Lee and Peart 1994, Alexandrou et al. 1992, Langenfeld et a. 1993 and Bowadt et al. 1994).

The clean-up step involves the separation of interfering compounds such as fat, sulphur and other co-extractants, which may interfere with the subsequent analysis, especially when using GC-MS (Erickson 1986). Moreover, the presence of fat deteriorates column performance and should therefore be eliminated to ensure accurate quantification of the PCBs.

After the clean-up a GC analysis is carried out usually without prior pre-separation according to planarity. However, the quantitative determination of coplanar PCBs would be difficult, since the non-planars are present in far higher concentrations and the resolution power of most GC columns is not sufficient to

separate all PCBs. Therefore, for the accurate determination of all PCBs a fractionation according to planarity is required. The analysis of non-and mono-ortho PCBs was reviewed by Hess et al. (1995) and the determination of non-ortho PCBs by Creaser et al. (1992). The fractionation in the groups according to their planarity was achieved by adsorption columns made from activated carbon (Atuma and Andersson 1993, Kannan et al. 1987a, Koistinen et al. 1993, Kocan et al. 1994) or Florisil (Lazar et al. 1992). HPLC columns such as porous graphitic carbon columns (Al-Haddad 1994) or 2-(1-pyrenyl) ethyldimethyl-silylated (PYE) silica column by Haglund et al. (1990) and dinitroanilinopropylsilica and tetranitroflouren-iminopropylsilica by Grimvall and Östman et al. (1994) were also applied to achieve the fractionation.

Ballschmiter et al. (1989) reported the retention behaviour of PCB congeners on several GC columns and suggested the use of a new n-octyl-methylpolysiloxane column, which separated the PCBs with regard to their chlorination level. These groups of PCBs having the same number of chlorines were further divided into subgroups which separated the PCBs according to their planarity and hence the column could be used for the determination of planar PCBs without an additional separation step, according to Ballschmiter (1989). The results after gas chromatographic separation using MSD or ECD allow the concentration of PCB to be expressed in various ways as discussed in detail by Erickson (1986).

3.4.2 Separation of PCBs According to their Planarity

Since some of the more toxic PCBs are present in concentrations which far exceeded that of TCDD, they have greater implications on the well-being of wildlife and humans. The biological activity of TCDD and PCBs involves the binding to the arylhydrocarbon (Ah)-receptor. The complex translocates to the cell's nucleus where it attaches to a specific sequence of the DNA, allowing attachment of other binding proteins and a transcription of cytochrome P450 or

other enzymes (Hanson 1991). The PCBs induce different proteins and their toxicity can be derived from the type of protein they induce and from the binding affinity to the Ah receptor. A summary of the measured activities was given by De Voogt et al. (1990). These studies allowed the estimation of the real impact of certain PCBs as the toxicity of the PCBs can be compared with that of TCDD. The determined level of the most toxic congeners can then be adjusted to TCDD equivalent concentration by the use the Toxic Equivalence Factor (TEF) developed by Sawyer and Safe (1982).

PCBs were separated according to their planarity on columns such as 2-(1-pyrenyl)ethyldimethylsilylated (PYE) silica column by Haglund et al. (1990) and on dinitroanilinopropylsilica and tetranitrofluoreniminopropylsilica by Grimvall and Östman et al. (1994). The retention mechanism in the PYE column was explained by a charge-transfer mechanism, in which the electron-density acceptor and donor regions of the PCBs induced a localisation change of the π-electron cloud of the stationary phase and formed an electron-donor-acceptor complex (Haglund et al. 1990). The degree of retention was explained by a) highly chlorinated PCBs forming stronger complexes with the pyrenyl moiety of the stationary phase, b) ortho substituted PCBs, which experienced a twisting of the biphenyl σ-bond due to steric hindrance and were less capable of forming strong complexes, hence were less retained and c) PCBs, in which the chlorines were closer together on one or both benzene rings, experienced higher retention, since they acted as stronger electron acceptors and therefore enhanced the strength of the complex (Haglund et al. 1990). Johansen et al. (1994) coupled SFE directly to a HPLC system equipped with a PYE column to allow the on-line separation of planar PCBs, which were subsequently analysed using GC-ECD or GC-MS. Moreover, basic alumina was added in a separate extraction cell which was coupled to the extraction cell to assist the removal of fat, so that a concomitant clean-up was achieved.

A similar retention mechanism was expected to take place in the (S)-NEC-β-CD column and the PCB retention was investigated at the following conditions: 40°C,

150kg/cm, 0.7ml/min flow CO_2. The peaks were detected at a wavelength of 225nm. A Rheodyne 7520 micro injector with an internal volume of 0.5µl was installed as this required less sample volume for rinsing than the previous installed Rheodyne 7413, which had additional tubing leading to the internal loop. The injection of 0.5µl of a 20-100ppm solution resulted in 10-50ng being injected onto the column.

Before analysing any PCBs on the SFC system, all connections were thoroughly checked to avoid any contamination of the system with PCBs. As there were no concentrated solutions of about 20-100ppm of the individual PCBs available in the laboratory, vials containing several mg of solid PCBs were dissolved completely in heptane. The preparation of accurately weighed solutions was avoided as weighing out the PCBs resulted in loss from the spatula due to electrostatic effects. Moreover, since no balance was accurate enough to weigh such small quantities, it was considered preferable to dissolve the remaining solid in the flasks and work only with an approximate concentration.

The structural formula of the PCBs and the numbering of the carbon atoms is given in Figure 3.26. Table 3.18 lists all the PCBs used in the present study according to the IUPAC numbering system, their Ballschmiter and Zell number and the calculated capacitiy factors.

Table 3.18 IUPAC structure, BZ number and retention factor of the analysed PCBs

BZ No.	IUPA nomenclature	Retention factor k[a]	Planarity	Toxicity[b]
Biphenyl	Biphenyl	2.365	planar	
PCB 1	2-Chlorobiphenyl	2.528	mono-ortho	
PCB 7	2,4-Dichlorobiphenyl	2.805	mono-ortho	
PCB 43	2,2',3,5-Tetrachlorobiphenyl	4.182	non-planar	
PCB 50	2,2',4,6-Tetrachlorobiphenyl	2.971	non-planar	
PCB 77	3,3',4,4'-Tetrachlorobiphenyl	10.613	planar	high TCDD like toxicity
PCB 80	3,3',5,5'-Tetrachlorobiphenyl	2.208	planar	
PCB 81	3,4,4',5-Tetrachlorobiphenyl	5.859	planar	
PCB 97	2,2',3',4,5-Pentachlorobipenyl	6.587	non-planar	
PCB 118	2,3',4,4',5-Pentachlorobiphenyl	7.315	mono-ortho	considerable toxicity
PCB 126	3,3',4,4',5-Pentachlorobiphenyl	9.961	planar	high TCDD like toxicity
PCB 143	2,2',3,4,5,6'-Hexachlorobiphenyl	4.227	non-planar	
PCB 156	2,3,3',4,4',5-Hexachlorobiphenyl	8.480	mono-ortho	
PCB 157	2,3,3',4,4',5'-Hexachlorobiphenyl	8.466	mono-ortho	considerable toxicity
PCB 169	3,3',4,4',5,5',-Hexachlorobiphenyl	9.358	planar	high TCDD like toxicity
PCB 183	2,2',3,4,4',5',6-Heptachlorobiphenyl	5.234	non-planar	
PCB 202	2,2',3,3',5,5',6,6'-Octachlorobiphenyl	3.658	non-planar	
PCB 207	2,2',3,3',4,4',5,6,6'-Nonachlorobiphenyl	4.129	non-planar	
PCB 209	2,2',3,3',4,4',5,5',6,6'-Decachlorobiphenyl	4.008	non-planar	

[a] Conditions: (S)-NEC-β-CD, 40°C, 150kg/cm^2, 0.7ml/min CO_2 flow.
[b] Toxicity according to Parkinson et al. 1983.

PCB 50 and 183 gave broad, tailing peaks which could not be readily explained, however since the PCB solutions were at least 3 years old, degradation could have occurred. This however was most unlikely due to the inherent stability of PCBs (Ballschmiter et al. 1989). One of the greatest problems with PCBs in the environment is due to their stability. PCBs degrade only very slowly and thus accumulate not only in the environment, but also in the adipose tissue of humans. Since the most toxic PCBs are more difficult to metabolise, an enrichment of these takes place. Figure 3.28 shows the retention factor in graphical form and it can be seen that a separation of the PCBs according to their planarity was achieved.

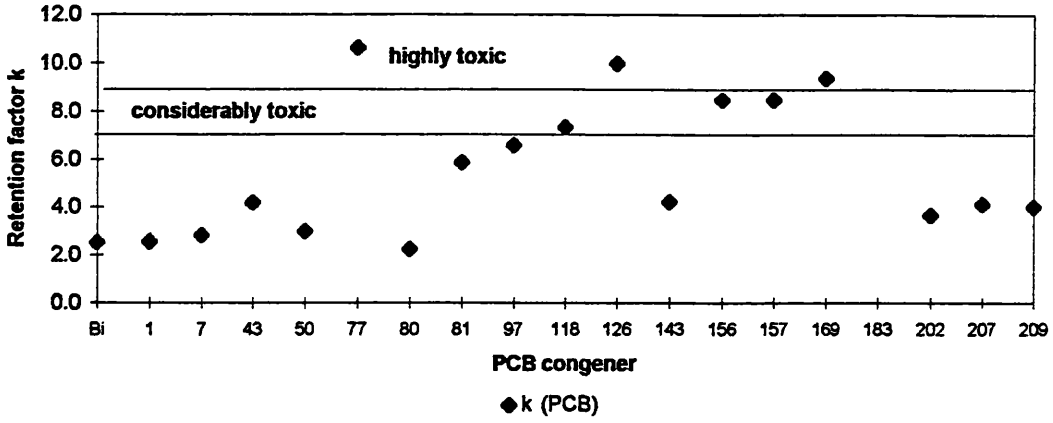


Figure 3.28 Separation of PCBs on a (S)-NEC-β-CD column according to Table 3.18.
Conditions: (S)-NEC-β-CD, 40°C, 150kg/cm^2, 0.7ml/min CO_2 flow, 225nm.

The PCBs 77, 126 and 169 are planar due to the absence of Cl-substitution on the ortho-position, and therefore were able to form the strongest charge-transfer complexes, which in turn resulted in the longest retention. PCB 81 also fulfils the requirement of being planar, however it appears to be less toxic (De Voogt et al. 1990) and as seen from Figure 3.28, eluted earlier than the non-planar PCB 97. The next group can be identified as the mono-ortho substituted, to which PCBs 105, 114, 118, 123, 156, 157, 167 and 189 belong, however only PCB 118, 156 and 157 were available in this study. PCB 118 eluted very closely to PCB 97, so that further optimisation of the conditions was necessary. The remaining PCBs which had a considerably shorter retention time, are with the exception of PCB 80 non-planar, and therefore eluted according to their toxicity. There were no reports regarding the toxicity of PCB 80, however low toxicity was expected due to the low retention on the (S)-NEC-β-CD column. This work demonstrates the novel use a (S)-NEC-β-CD column in SFC to achieve a separation of PCBs according to their toxicity. Camman and Kleiböhmer (1990, 1991) separated PCB mixtures on cyanopropyl and ODS columns. The PCBs were separated primarily into groups of homologues, having the same chlorine atom number and secondly according to their planarity as observed by Ballschmiter et al. (1989) on a GC column. However, there was no

distinct separation of planar PCBs which would allow this separation as a pre-separation step.

As can be seen in Figure 3.28, PCB 156 eluted very closely to PCB 169 and PCB 97 to PCB 118, and therefore the resolution of PCB 97 : 118 and PCB 156 : 169 was optimised with a view to enable the collection of fractions, as this was the ultimate aim for the separation according to planarity. Figure 3.29 shows the separation of these pairs under the above conditions.

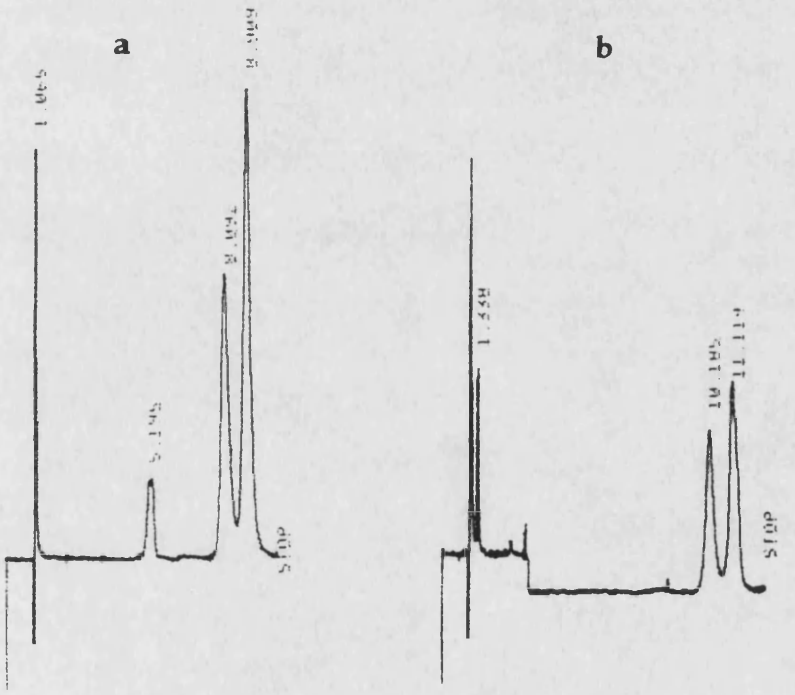


Figure 3.29 Separation of a) PCB 97 : 118 and b) PCB 156 : 169 on a (S)-NEC-β-CD column.
Conditions: (S)-NEC-β-CD, 40°C, 150kg/cm^2, 0.7ml/min CO_2 flow.

The temperature was increased to 50°C to see whether this improved the separation. It either could have deteriorated the separation due to an increase in

thermal energy, hence disrupting the interaction between stationary phase and analyte or improved the resolution due to enhancing the solubility differences at lower density. A further reduction of the pressure to 140kg/cm^2 resulted in a sufficient separation to allow the collection of the different fractions. Figure 3.30 demonstrates the improved separation of PCB 97 :118 and PCB 156 : 169 on the (S)-NEC-β-CD column.

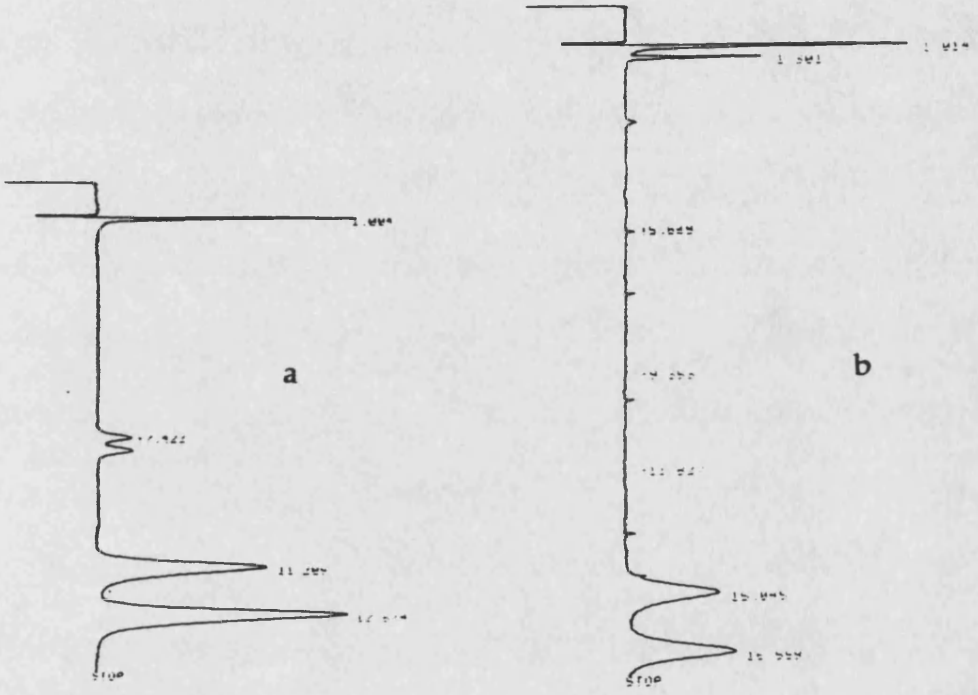


Figure 3.30 Optimised Separation of a) PCB 97 : 118 and b) PCB 156 : 169 on a (S)-NEC-β-CD column
Conditions: (S)-NEC-β-CD, 50°C, 140kg/cm^2, 0.7ml/min CO_2 flow.

Fractionation of PCBs: Determination of the toxic planar PCBs, being present at ultra-trace levels compared to that of non-planar PCBs, imposes a very difficult task on analytical chemists, therefore it is necessary to separate the different groups to allow accurate quantification.

Initial trials of fractionation were conducted using PCB 97 to see whether the PCB was efficiently collected in 5ml of heptane (fraction 1.1) within 30 seconds after peak elution. The PCB had a concentration of about 80ppm which allowed the peak elution to be monitored via UV detection at 225nm. Before the trial was conducted an additional pump was linked between the column exit and the detector via a stainless steel T-piece to enable the flushing of the lines through the UV detector and more importantly through the backpressure regulator and the exit tubing. The SFC system was kept under pressure by means of the backpressure regulator and a needle valve, which maintained the pre-set pressure by opening and closing the valve at regular intervals. This caused the fluid mixture to depressurise and may have caused the PCBs to drop out of solution within the valve and the 10cm long tubing and prevent a quantitative transfer to the collection vial. The ease of separation of solute and solvent, expressed as the ratio of $P_{v,\,sat\,solvent}$/ $P_{v\,sat}$, was cited as one of the advantageous of SCFs by Lee and Markides(1990).

The additional pump therefore enabled the flushing of the lines with a mixture of heptane : acetone (1:1) once PCB 97 had been collected. The lines and backpressure regulator were rinsed with heptane : acetone (1:1) at a flow rate of 0.3ml/min for 15 minutes without a CO_2 flow and the rinsing was collected in a new collection vial (fraction 1.2). Moreover, the column was also rinsed for 15 minutes with CO_2-15% 2-PrOH at a flow rate of 0.7ml/min to ensure that no irreversible adsorption took place on the column (fraction 1.3). The ECD analysis (described in section 2.2.7) of the different fractions revealed that only 55% was trapped in fraction 1.1, which was most likely due to the precipitation of the PCB in the needle valve and the subsequent tubing. This was confirmed by the analysis of the fraction 1.2 which proved that about 35% PCB 97 remained in the backpresure regulating valve and subsequent tubing. The analysis of the column rinse did not reveal the presence of any PCB 97, so that irreversible adsorption could be excluded and thus the loss was mainly due the inefficient transfer of the PCB into

the collection vial. Incomplete transfer from the collection tube to the analyses vial was eliminated by evaporating the solvent almost completely and then rinsing the tube with additions 2ml of solvent and repetition of the evaporation step. The final volume was made up to 2ml. Campbell (cited in Porter et al. (1992) noted the need for rinsing the flow meter control valve and the attached tubing in order to obtain quantitative recoveries of PCBs. The valve in which decompression took place, contained significant concentrations of precipitated PCBs. Moreover, Bowadt et al. (1989) quantified the loss in a transfer line for PCBs and observed significant %losses depending on the chlorination level of the PCBs. The recovery for the PCB 97 in this work however was only evaluated on a semi-quantitative basis, as this initial trial was supposed to be a confirmation of the assumption that PCBs remain in the tubing and backpressure regulator. For an accurate quantification ^{13}C labelled isotopes would be necessary, as described by Haglund et al. (1990). Mills and Jefferies (1993) reported the separation of PCBs from fat using SFC on a polymeric column and indicated an approximate recovery of 60%, despite using a high flow rate of 2.3ml/min of CO_2 mixture containing 20% 2-PrOH, which would suggest that sufficient rinsing took place. However, since the collection of subsequent fractions did not contain any PCBs the losses in that investigation must have occurred at a different stage.

It was then decided to leave the additional pump (pump 3) running during the collection of the fractions to ensure the quantitative transfer to the collection trial. The collection volume of heptane was reduced to 3ml, as the addition of 0.3ml/min of heptane : acetone mixture from pump 3 increased the collection volume. The collection was monitored via a UV-detector, which required a concentration of PCBs of about 80ppm, in order to monitor the elution of the peaks so as the collection vial could be changed accordingly. The solution of PCB 118:97 was made up by diluting the original PCB 118 (~1.5mg/ml) 1:20 and a solution of PCB 97 (0.5mg/ml) by 1:6 in the same solution so that the final concentration of both PCBs was about 80ppm, which resulted in the injection of about 40ng of both PCBs onto

the column. The final concentration injected on the ECD was 20ng/ml as the final solution of the PCB was contained in 2ml. The additional pumping caused the baseline to become very noisy. Nevertheless, it was still possible to observe the separation as seen in Figure 3.31.

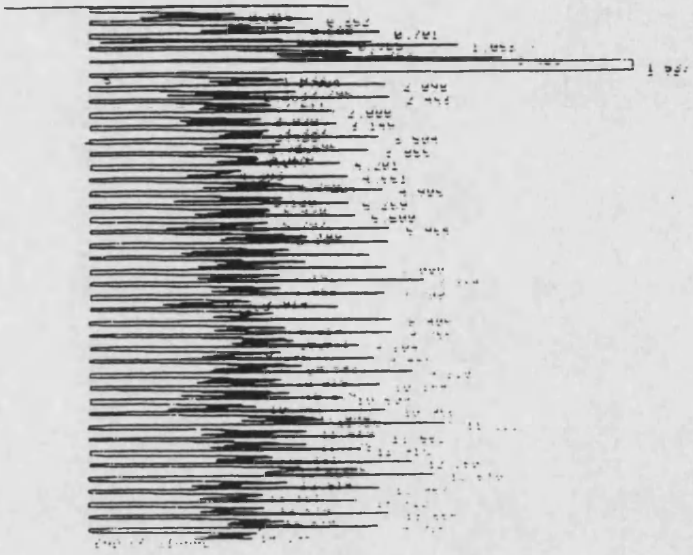


Figure 3.31 Chromatogram showing the separation of PCB 97 :118 when additional solvent was added after the column and before the UV detection.
Conditions: (S)-NEC-β-CD, 50°C, 140kg/cm^2, 0.7ml/min CO_2 flow, 0.3ml/min heptane : acetone for tubing flushing.

The collection vial was changed after about 11.8mins (fraction 2.1) and the next fraction (PCB 118) was collected until 15mins, which was 2mins longer than the elution of the peak (fraction 2.2). Another fraction was collected for another 10mins (fraction 2.3) at the conditions of fraction 2.2 in order to determine whether the quantitative transfer was achieved. After the fraction collection (2.1, 2.2 and 2.3) the column was rinsed for 15 min to investigate if irreversible adsorption took place. The GC-ECD analysis of the compounds revealed that there were still carry-over effects.

As seen from the Table 3.19 there were still PCBs present in the column fraction, however it was more likely that this resulted from PCBs which had remained in the tubing. The initial trial with PCB 97 showed that rinsing the tubing and backpressure regulator with solvent alone resulted in a PCB free column fraction. This indicated that the additional rinsing of the tubing and backpressure regulator with heptane : acetone during the fractionation of PCB 97 : 118 was not sufficient to ensure a quantitative transfer, which was also confirmed by the presence of 19% of PCB 97 in the second fraction in Table 3.19.

Table 3.19 Distribution of PCB 97 : 118 in the collected fractions

Fraction	Descripton of fraction	PCB 97 distribution [%]	PCB 118 distribution [%]
2.1	PCB 97 collection until 11.5min	74.5	–
2.2	PCB 118 collection from 11.5-14min	19	94
2.3	collection from 14-24min	4.5	4.5
2.4	column rinse CO_2 + 15% MeOH	2	1.5

The carry-over effects therefore posed a severe problem on the quantitative collection of the separate fractions, however they were thought to be exclusively due to the design of the instrument set-up.

Saito and Yamauchi (1990) reported the development of the novel backpressure regulator, which has a dead-volume of less than 10µl, and demonstrated its use for fractionation of cold-pressed lemon peel oil. They also added a third pump to flush the backpressure regulator and tubing to assist the quantitative transfer of volatile compounds, even when a flow rate of 2.2ml/min CO_2 was used. In this study a flow rate of only 0.7ml/min CO_2 was applied, however such a flow rate created additional and more problematic conditions, which made the quantitative transfer even less efficient. The flow rate in this study was determined by the application of 2.1mm i. d. columns. These were used with a view of connecting the SFC directly to a GC without

flow splitting, allowing higher concentration to be transferred onto the column, hence lowering detection limits.

An Aroclor 1260 at a concentration of 1093ppm in heptane was used for the fractionation trial, in which 2 fractions were collected. The first fraction was collected from 0-11.5minutes (fraction 3.1) in order to collect the ortho-substituted PCB'c and a second fraction for the mono-ortho and planar PCBs (fraction 3.2) from 11.5-25mins. After the fraction collection the column was rinsed again with a mixture of 15% MeOH-CO_2 mixture, to estimate the residue on the column, tubing and backpressure regulator. The analysis of the individual fractions showed that a fractionation took place, however there were also carry-over effects. Figure 3.32 a-c show the original Aroclor mixture and the collected fractions 3.1 and 3.2, respectively.

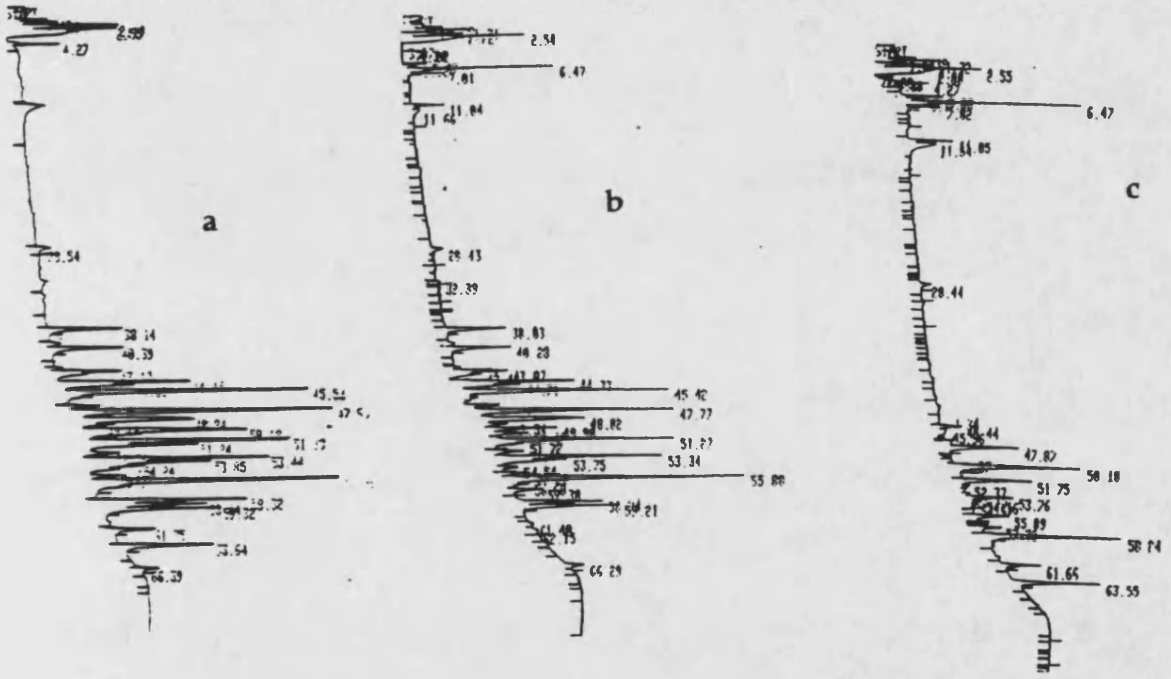


Figure 3.32 Chromatogram of a) original Aroclor mixture 1:3600 diluted, b) fraction 3.1 and c) fraction 3.2.

GC-Conditons: HP 5895 GC-ECD, BPX 5 column (25m, 0.2mm i. d., 0.22μm film th.), on-column injection, 1.2ml/min He (carrier gas), 50°C, 2min, 20°C/min, 150°C, 10min, 2°C/min, 250°C, 5min, 350°C detector temperature, 30ml/min N_2 (make up gas).

From the chromatograms it can be clearly seen that the first two peaks at 38 and 40 minutes were completely collected in fraction 3.1 with about 80% efficiency. Almost all the peaks up to 47 min were found in fraction 3.1, however carry-over effects were observed for the peaks at 45 and 47 minutes. Nevertheless, there were several peaks which could only be identified in fraction 3.2 and not the first fraction. The peaks at 58.24, 61.66 and 63.55min were solely found in fraction 3.2, the peaks at 50.10 and 51.75min were mainly found in fraction 3.2, but the remaining peaks were found in both fractions. By spiking fraction 3.2 with the available PCBs (81, 118, 156, 157 and 77, 126, 169) only minor amounts of PCB 118 and PCB 156 could be identified. Published results of concentration of these PCBs in Aroclor 1260 as listed in Table 3.20 show that only PCB 118 and 156 are present at a significant level. It is therefore not surprising that no distinctive peaks were detected for the most of the listed PCBs, since the fractionation diluted the PCBs by 1:4000. Moreover, there appears to be a wide variation in the level of PCB 77, 126 and 169 in commercial mixtures, for example Hess et al. (1995) reported the concentration of these PCBs in a Aroclor 1260 mixture to be below the detection limit.

Table 3.20 Presence of mono-ortho and planar substituted PCB congeners in Aroclor 1260

Congener	Aroclor 1260	
	a) (µg/g)	b) (µg/g)
105	800	—
114	—	—
118	10000	—
123	—	—
156	10000	—
157	700	—
167	1500	—
189	3500	—
77	<100	270
81	<100	—
126	<100	5
169	500	<0.08

[a] from Duinker et al. (1988), [b] from Kannan et al. (1987).

The solution of fraction 3.2 was thus concentrated 10 times and analysed. The resultant chromatogram is shown in Figure 3.33.

Comparing the chromatogram in Figure 3.33 with a spiked chromatogram, the PCB congeners 81, 118, 126 and 156 could be identified. However, at the same time the presence of 8 major PCB peaks could be observed, suggesting that the separation using (S)-NEC-β-CD column was not purely according to planarity like the fractionation achieved by either the PYE (Haglund et al. 1990) or dinitroanilinopropylsilica by Grimvall and Östman (1994). However, it would be necessary to identify the peaks by retention time comparison in order to fully appreciate the separation achieved with the (S)-NEC-β-CD column. Furthermore, PCBs could still be detected in the column rinse, although this was likely to stem from the flushing of the backpressure regulator while rinsing the column.

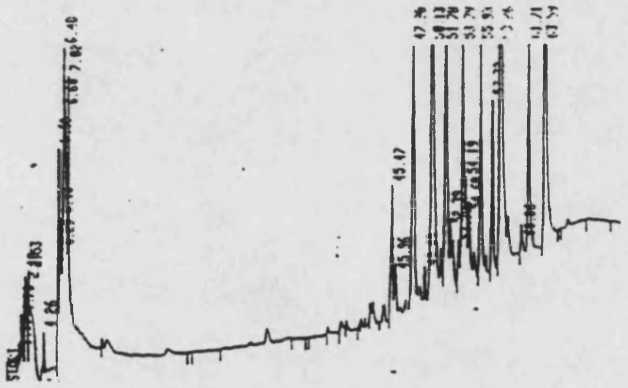


Figure 3.33 Chromatogram of concentrated fraction 3.2.
GC-Conditons: as before in 3.31.

The above trials demonstrated that a (S)-NEC-β-CD column can be used for the partial separation of PCBs according to planarity, however the retention behaviour of a greater number of individual PCBs should be investigated for a better understanding of the retention mechanism. At the same time the problems of quantitative transfer of the compounds into the collection vial were highlighted.

For the collection of compounds present in trace concentration, it would be recommended to ensure a direct transfer onto the GC, thus avoiding problems of transfer and dilution of the compounds, by using the approach of Wright et al. (1987).

3.3.3 Retention behaviour of PCB congeners on different columns

It was envisaged to develop a method with which the affinity of PCB congeners with the Ah receptor would be modelled. McKinney et al. (1983) developed a method for calculating polarisability, since their model of PCB binding to the cytosol receptor was based on polarisabilities and binding distances of receptor to PCB. They found good correlation for $EC_{50,i}$ determined by Bandiera et al. (1982) with the calculated equilibrium constant obtained from dispersion forces using porphine as a model receptor. $EC_{50,i}$ indicates the concentration of PCB necessary to reduce the binding of [^{3}H]TCDD to 50% on the Ah receptor and therefore represents binding strength. This model was extended to include related compounds exerting other electronic effects other than dispersion in order to test its applicability (McKinney et al. 1985). It was, observed, however that compounds possessing substituents which were able to exert forces such as dipole-dipole, electronic charge transfer, electrostatic, resonance stabilisation and covalent binding could not be accurately modelled with the suggested model (McKinney et al. 1985).

Calculations of retention factor ratios were performed on the values obtained from the (S)-NEC-β-CD and the β-CD, diol and silica column with the aim that the calculated ratios give an indication of the enhanced retention on the (S)-NEC-β-CD column due to charge-transfer complexes or dispersion forces. This ratio might then be a guide to the interaction strength of PCBs with the Ah receptor. The solubility effect of decreasing solubility with increasing chlorination should be

eliminated by the calculated ratio, hence it should solely express the enhancement of retention on the (S)-NEC-β-CD column.

The retention on the (S)-NEC-β-CD column was compared to a β-CD column, diol and silica column at 50°C, 140kg/cm^2 and a flow rate of 0.7ml/min. Table 3.21 lists the retention factor obtained on each column and Table 3.22 indicates the calculated ratios of (S)-NEC-β-CD to the remaining columns.

Table 3.21 Retention factors of PCB congeners on different columns[a]

BZ No.	Retention factor k			
	(S)-NEC-β-CD	β-CD	Diol	Silica
Biphenyl	2.987	1.368	0.804	0.848
PCB 1	3.269	1.508	1.032	0.846
PCB 7	3.903	1.719	1.098	0.792
PCB 43	6.313	2.842	2.098	1.029
PCB 50	3.107	2.309	1.655	0.821
PCB 77	16.184	5.506	2.541	1.224
PCB 80	3.673	1.464	1.260	0.659
PCB 81	9.470	2.987	2.005	1.000
PCB 97	9.811	4.005	2.468	1.110
PCB 118	11.710	3.954	2.029	0.968
PCB 126	15.960	4.001	2.692	1.208
PCB 143	6.961	3.351	3.043	1.200
PCB 156	14.053	3.907	2.698	1.146
PCB 157	13.911	4.467	3.208	1.317
PCB 169	15.651	3.683	2.745	1.269
PCB 183	5.234	3.994	2.191	0.915
PCB 202	6.461	2.847	2.484	0.937
PCB 207	7.579	3.035	2.819	1.009
PCB 209	7.579	3.140	3.073	1.037

[a] Conditions: 50°C, 140kg/cm^2, 0.7ml/min CO_2 flow rate, 225nm.

As can be seen from Table 3.22 and Figure 3.34, there is an enhancement for the mono-ortho and non-ortho PCBs, however they do not correlate with the binding affinities measured by Bandiera et al. (1982), which stated that PCB 126 was the

most toxic. The enhancement for PCB 81, which showed less toxicity, has a relatively high ratio of (S)-NEC-β-CD : β-CD.

Table 3.22 Retention factor ratios of PCB on (S)-NEC-β-CD compared to β-CD, Diol and Silica

BZ No.	Retention factor k		
	(S)-NEC-β-CD : β-CD	S)-NEC-β-CD : Diol	S)-NEC-β-CD : Silica
Biphenyl	2.18	3.72	3.52
PCB 1	2.17	3.17	3.86
PCB 7	2.27	3.55	4.93
PCB 43	2.22	3.01	6.14
PCB 50	1.35	1.88	3.78
PCB 77	2.94	6.37	13.22
PCB 80	2.51	2.92	5.57
PCB 81	3.17	4.72	9.47
PCB 97	2.45	3.98	8.84
PCB 118	2.96	5.77	12.10
PCB 126	3.99	5.93	13.21
PCB 143	2.08	2.29	5.80
PCB 156	3.60	5.21	12.26
PCB 157	3.11	4.34	10.56
PCB 169	4.25	5.70	12.33
PCB 183	1.31	2.39	5.72
PCB 202	2.27	2.60	6.90
PCB 207	2.50	2.69	7.51
PCB 209	2.41	2.47	7.31

The problem with the ratio of (S)-NEC-β-CD : β-CD was that inclusion complexes are considered to take place on the β-CD column, although not on the (S)-NEC-β-CD column. The ratio did therefore not solely reflect the enhancement due to interaction with the aromatic moiety of the (S)-NEC-β-CD column.

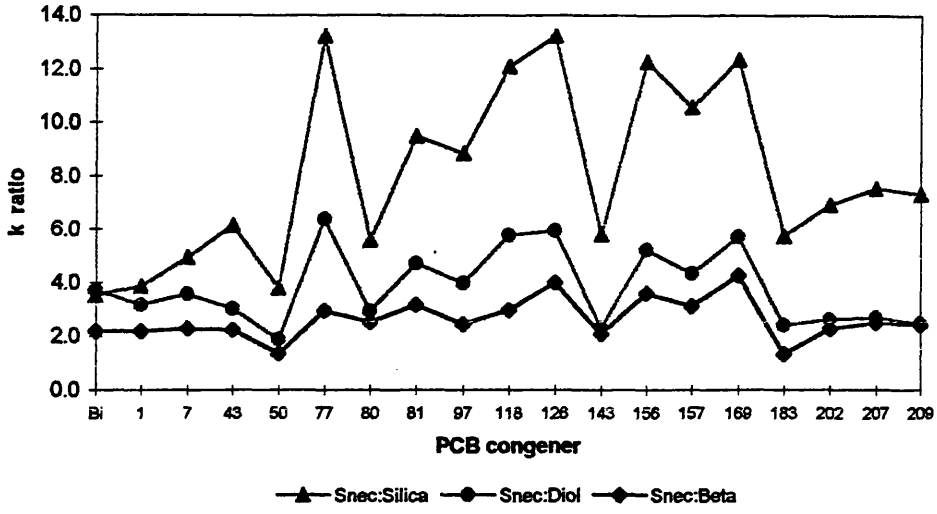


Figure 3.34 Calculated ratio of retention factors between (S)-NEC-β-CD column and β-CD, Diol and Silica
Conditons: 50°C, 140kg/cm^2, 0.7ml/min CO_2 flow rate, 225nm.

The ratio of (S)-NEC-β-CD : Silica appeared to correlate best with toxicity, as PCB 81 obtained a lower ratio than PCB 157 and PCB 77 a higher ratio than PCB 118. There were however still deviations observed (the value for 77 compared to 126 was too high and that for 157 too low) and the same was observed with the (S)-NEC-β-CD : Diol ratio. Moreover, there were doubts whether the (S)-NEC-β-CD column was necessarily the best model for the Ah receptor, since the micro-environment of the receptor was supposed to contain a tryptophan group (McKinney et al. 1985). It would therefore be very interesting to produce a derivatised cyclodextrin and silica column, which contains a tryptophan moiety to mimic the Ah receptor. By calculating the ratio of enhancement on these newly developed columns with yet to be identified other columns, it should be possible to estimate the strength of the interaction of compounds with the Ah receptor. For every column combination ratios would be identified above which compounds could be identified as being toxic. For the combination (S)-NEC-β-CD : β-CD the value would be set to 2.9, for the (S)-NEC-β-CD : Diol to 4.3 and for the (S)-NEC-β-CD : Silica to 10, since this includes the toxicity level of the mono-ortho substituted PCBs. This model, however suffers the same limitations as the computer model of

McKinney (1984) as only the bonding affinity would be determined. The action of TCDD is far more complex than the sole binding to the cytosolic Ah receptor (Poland and Knutson 1982, Eisen et al. 1983). Lu et al. (1996) demonstrated that binding affinity cannot be directly correlated to toxicity in the case of substituted flavones, but nevertheless confirmed that there is a structure-activity relationship for the PCBs. To sum up, the calculated ratios for the PCBs indicated different degrees of binding affinities, however it would not be possible to extend this model to unknown compounds and deduce toxicity from the calculated ratios, since binding affinity does not necessarily correlate to toxicity for all compounds. Nevertheless, it may be worthwhile to investigate the retention behaviour of the substituted flavones investigated in the study of Lu et al. (1996).

Moreover, there has been a number of reports discussing the effect of chemicals on the fertility of humans and other animals. PCBs were listed as one of the chemicals which disrupt the endocrine system (Lee 1996 and De Voogt 1990). It would therefore be beneficial if the newly developed column or (S)-NEC-β-CD column would not only indicate the toxicity of PCBs, but also correlate to compound's endocrine disrupting properties. This method could be also considered for closer investigation, as numerous compounds could be studied in a very short time. In addition, if the use of cyclodextrin columns were considered, it would also be possible to use mobile phases other than SCF, as CD are stable under both RP and NP conditions, extending the applicability of this measurement. In section 3.4.6 there are additional calculations for a variety of compounds in order to test the proposed model.

3.4.4 Separation of PCBs from Fat

Due to the hydrophobic nature of PCBs, an accumulation of these compounds takes place in the adipose tissue of mammals. The separation of the fat from the

PCBs prior to gas chromatographic analysis is crucial, since it deteriorates the chromatographic performance significantly (Erickson 1986).

The elution of PCB congeners on various stationary phases has been demonstrated in section 3.3.3 and the retention range of the PCBs on each column is collated in Table 3.23.

Table 3.23 Retention range of PCB congeners on various stationary phases

	Retention factor k on different stationary phases			
PCB	**(S)-NEC-β-CD**	**β-CD**	**Diol**	**Silica**
first eluting	3.27 (1)[a]	1.51 (1)	1.03(1)	0.85(1)
last eluting	16.18 (77)	5.51 (77)	3.21(157)	1.32(157)

[a] the number in brackets indicates the PCB congener.
Conditions: 50°C, 140kg/cm², 0.7ml/min.

The elution of fat was then investigated under the same conditions on each of the columns, in order to judge whether any of the above columns allowed the separation of the PCBs from the fat. Witepsol S55 and sunflower oil were used for the investigation of the elution behaviour of fat, since Witepsol S55 has a similar composition to milk fat and the analysis of PCBs in human and cows milk is frequently required. Both the sunflower oil and Witepsol S55 did not elute from any of the above columns under 30 minutes, with the exception of sunflower oil which started eluting on the diol column at 25 minutes. The results therefore indicated that each of the columns can be used for the separation of PCBs from the fat. Additionally, it was advantageous that the PCBs eluted before the fat, so that even when the column was overloaded, there would still be sufficient separation. As seen from the table the elution of PCB congeners on the (S)-NEC-β-CD column took place over a long retention time range and therefore was not considered further for the separation of PCBs from fat. β-CD, diol and silica however, all appeared suitable and provided a fast separation, however the use of the silica or diol column was favoured due to their low cost.

As the PCBs were eluted under the same conditions necessary for the fractionation, the same problem of quantitative transfer would be encountered, therefore no collection efficiency was determined.

3.4.5 Analysis of Witepsol S55

In order to elute Witepsol and sunflower oil from the columns, the following conditions were investigated: 50°C, 140kg/cm^2, CO_2 + 5% 2-PrOH, 0.7ml/min total flow. The separation on each of the columns can be seen in Figure 3.35 - 3.36. This again demonstrates the versatility of SFC and in particular of the chiral (S)-NEC-β-CD and β-CD column.

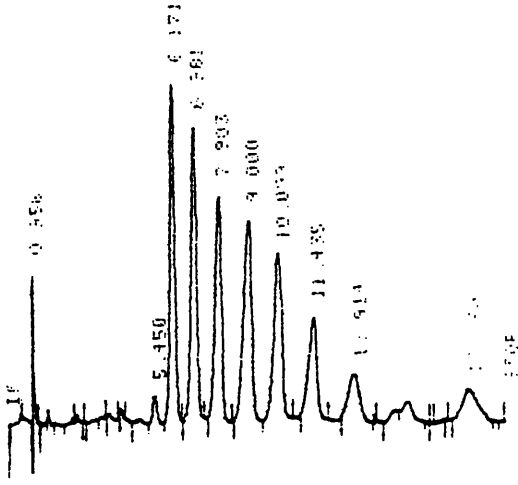


Figure 3.35 Separation of Witepsol S55 on a (S)-NEC-β-CD column.
Conditions: 50°C, 140kg/cm^2, CO_2 + 5% 2-PrOH, 0.7ml/min total flow, 225nm.

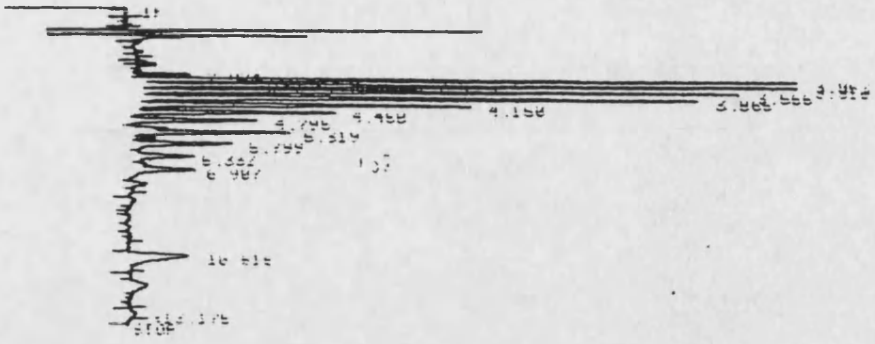


Figure 3.36 Separation of Witepsol S55 on a β-CD column
Conditions: 50°C, 140kg/cm^2, CO_2 + 5% 2-PrOH, 0.7ml/min total flow, 225nm.

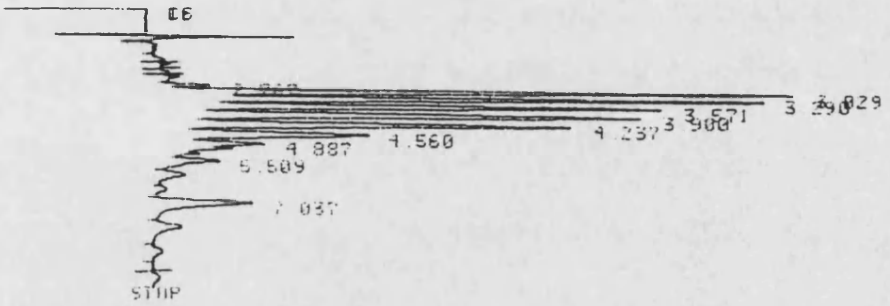


Figure 3.37 Separation of Witepsol S55 on a Diol column.
Conditions: 50°C, 140kg/cm^2, CO_2 + 5% 2-PrOH, 0.7ml/min total flow, 225nm.

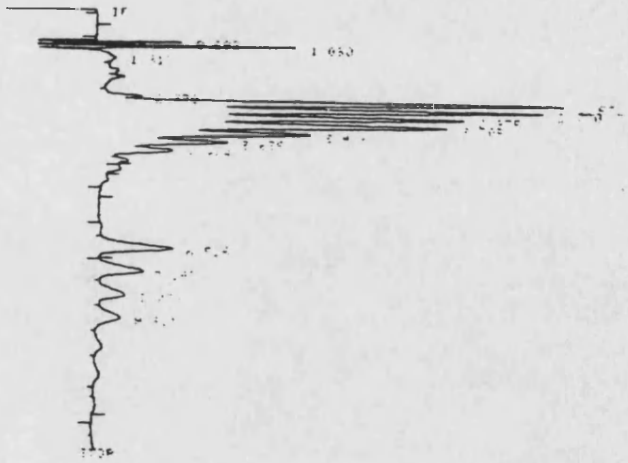


Figure 3.38 Separation of Witepsol S55 on a Silica column.
Conditions: 50°C, 140kg/cm^2, CO_2 + 5% 2-PrOH, 0.7ml/min total flow, 225nm.

As the separation of Witepsol S55 on the (S)-NEC-β-CD was relatively slow in comparison to separation on the other columns, the separation was conducted under the same conditions, however 2-PrOH was replaced by MeOH. Figure 3.39 shows the improved separation.

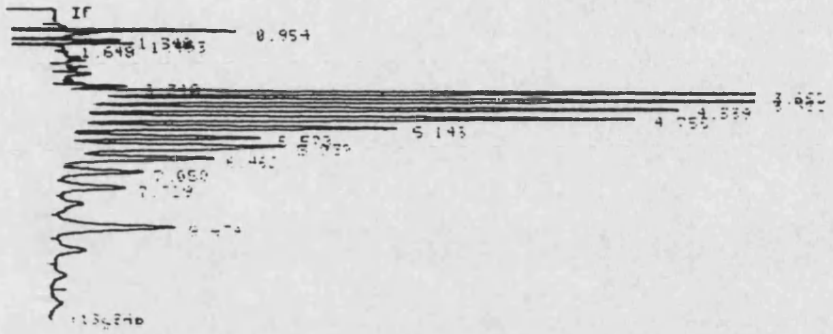


Figure 3.39 Separation of Witepsol S55 on a (S)-NEC-β-CD column.
Conditions: 50°C, 140kg/cm^2, CO_2 + 5% MeOH, 0.7ml/min total flow, 225nm.

Additionally, Witepsol S55 consists of glycerides of saturated fatty acids and therefore the separation of Witepsol on an ODS column was considered to be worthwhile investigating. As the ODS column had an inner diameter of 4.6mm i.d., a total flow rate of 3.0ml/min was used. MeOH was used as modifier since 2-PrOH gave a similar result to that on the (S)-NEC-β-CD column. Figure 3.40 represents the separation of Witepsol S55 on an ODS column using 3% MeOH as modifier.

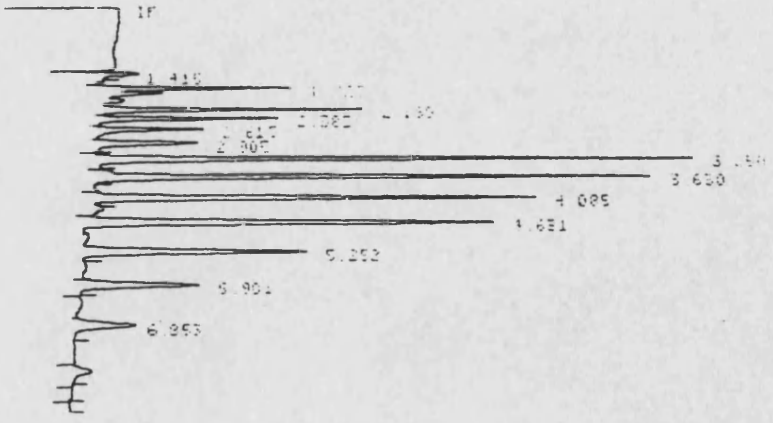


Figure 3.40 Separation of Witepsol S55 on a ODS column.
Conditions: 50°C, 140kg/cm^2, CO_2 + 3% 2-MeOH, 3.0ml/min total flow, 225nm.

3.4.6 Separation of PAHs from Fat

The presence of PAHs in edible oils and the formation of PAHs during the cooking process results in considerable amounts being released into the atmosphere (Shuguang et al. 1994). This was correlated with the high incidence of lung cancer in Chinese women. It was therefore vital to detect trace amounts of PAHs in oil.

The retention behaviour of the of the EPA (Environmental Protection Agency) 16 priority PAHs was investigated on different columns, to confirm whether the same kind of separation could be achieved as that of the PCBs. Table 3.24 lists the elution behaviour of PAHs, Witepsol S55 and sunflower oil on (S)-NEC-β-CD, β-CD, diol and silica.

Table 3. 24 Retention behaviour of PAH and fat on different columns

	Retention time of last eluting PAH [min]			
	(S)-NEC-β-CD	β-CD	Diol	Silica
Witepsol S55	>40	>19	>30	>30
Sunflower oil	>40	>30	11.8	>30
PAHs	33	17	16	5.5

Conditions: 40°C, 125kg/cm^2, 1.0ml/min CO_2, 225nm for fat detection, 254nm for PAHs.

The results demonstrated that a separation of PAHs with the silica column was the most efficient, since a separation was achieved within less than 10 minutes. Heaton et al. (1994b) investigated the separation of PAHs using SFC on a special column for PAH. The fat could therefore be separated on the silica column and the PAHs fraction heart cut to the PAH column used by Heaton et al. (1994b) for quantification.

3.4.7 Evaluation of Toxicity and Endocrine Disrupting Property

The idea of modelling the toxicity and/or the ability to disturb the endocrine system in mammals was further tested by calculating the ratios of the compounds investigated in this thesis. In addition the ratios of 3-hydroxy-PCBs were also investigated. Hydroxy-PCBs are formed by the metabolising process of PCBs and are considered to be less toxic (Safe 1989). Table 3.25 lists the calculated ratios for the hydroxy-PCBs and the ratios considered to represent toxicity comparable to that of mono-ortho chlorinated PCBs. The hydroxy-PCBs can be considered to be less toxic, as they are below the determined values as listed in Table 3.25.

Table 3.25 (S)-NEC-β-CD ratio of hydroxy-PCB calculated from retention factors on (S)-NEC-β-CD, β-CD, diol and silica columns

	Ratio		
	(S)-NEC-β-CD : β-CD	S)-NEC-β-CD : Diol	S)-NEC-β-CD : Silica
Toxicity ratio of mono-ortho PCBs	2.9	4.3	10
PCB 43-OH (1)[a]	1.82	1.96	4.54
PCB 77-OH (2)[b]	1.77	2.36	8.72
PCB 169-OH (2)[c]	1.53	1.88	5.24

Conditions: 40°C, 150kg/cm^2, 0.7ml/min CO_2 + 15% MeOH, 225nm.
[a] 43-OH: 2,5,3'-trichloro-biphenyl-2'-ol, 77-OH: [b] 4,4'-diichloro-biphenyl-3,3'-diol.
[c] 169-OH: 3,3',5,5'-tetrachloro-biphenyl-4,4'-diol.

The same calculations were then conducted for the alkaloids, propanolol and clofibrate analogues, Bn-ether and additionally for 5 phthalates. The results can be seen in Table 3.26.

Table 3.26 Calculated ratios of various compounds for the estimation of toxicity and endocrine disruption

	Ratio		
Compound	(S)-NEC-β-CD : β-CD	(S)-NEC-β-CD : Diol	(S)-NEC-β-CD : Silica
Limit of mono-ortho PCB	2.9	4.3	10
Nicotine	1.75	2.12	1.06
Cotinine	2.06	2.62	1.66
Anabasine	1.89	3.11	1.60
Nornicotine	1.57	2.48	2.57
Compound	(S)-NEC-β-CD : β-CD	Compound	(S)-NEC-β-CD : β-CD
C1	1.33	P1	2.15
C2	1.26	P2	2.06
C3	1.40	P3	2.16
C4	1.33	P4	1.90
C5	1.30	P5	2.20
C6	1.69	P6	2.11
C7	1.87		
C8	1.75	Phthalates	
C9	1.32	Dimethyl	2.96
C10	1.34	Diethyl	2.96
C11	1.67	Dibutyl	3.5
C12	1.99	Benzoyl	3.93
		Dioctyl	3.93
Bn-ether	2.26		

The ratios for (S)-NEC-β-CD : Diol and (S)-NEC-β-CD : Silica could not determined for all compounds, as some of the separations were only conducted on (S)-NEC-β-CD and β-CD columns. The results of the (S)-NEC-β-CD : β-CD ratio indicated that all the compounds with the exception of the phthalates should not interact with the Ah receptor and the hormone receptor, since the limit of the toxic ratio of 2.9 is not exceeded. However, it is unlikely that the phthalates interact with the Ah receptor, as no toxicity has been reported for these compounds.

The results suggested that the calculated ratios may be more suited to estimate a compounds' endocrine disrupting properties than for the estimation of toxicity caused by Ah receptor interactions. This was expected, as there have been publications regarding the failure to correlate Ah receptor binding coefficients with toxicity (Lu et al. 1996). The validity of the model to test endocrine disrupting properties has to be further corroborated by investigating the retention behaviour of the compounds listed by Lee et al. (1996).

The ratios for the alkaloids using the diol and silica column also suggested that the alkaloids do not fall into the category of endocrine disrupting chemicals, which indicated that the model may also be viable for a wide range of compounds.

CHAPTER 4

CHIRAL SFC RESULTS AND DISCUSSION

4.1 Introduction

There is an increasing demand for the enantiomeric separation of compounds, as a large proportion (50%) of pharmaceutical drugs are chiral, however only 10% of synthetic drugs are available as pure enantiomers (Parker 1991). As many pharmacological interactions are stereospecific, it is essential to assess the action of a chiral drug and its potential chiral metabolites on target organisms. Enantiomers may differ not only in their activity but also in their action, as in the case of thalidomide, whose teratogenic activity may reside exclusively in the S-isomer (Blaschke et al. 1979). If the possibility exists to chirally resolve an enantiomeric drug, then the behaviour of its enantiomers can be studied *in vivo* and the necessary data can be generated for market approval. For chiral separation there is a wide variety of chiral stationary phases available and CDs have found widespread application (Macaudiére et al. 1987).

4.1.2 β-CD Structure and Stereochemical Interaction

The three most common and naturally occurring CDs are α-, β- and γ-cyclodextrins being composed of 6, 7 and 8- α-D-(+) glucose units respectively. These glucose units, which are linked together by α-(1,4) linkages form a hollow toroid having a wider diameter on one end. On the wider opening, there are secondary hydroxyl groups in position 2 and 3 and on the narrower opening primary hydroxyl groups in position 6. The hydroxyl groups create a hydrophilic

character on the rim, which can be compared to a high density diol phase (Technicol 1992), whereas the CD cavity is relatively non-polar due to the presence of the glucosidic oxygen atoms. A patented process is applied to create an ether linkage of a specific length between the silica gel and one or two of the primary hydroxyl groups on the cyclodextrin moiety. Figure 4.1 shows the schematic outline of a cyclodextrin cavity bonded to a silica surface.

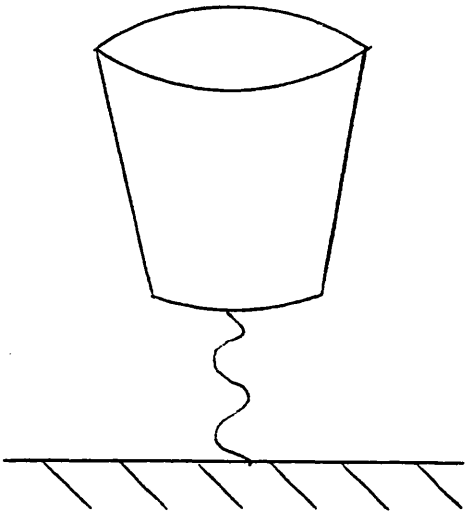


Figure 4.1 Cyclodextrin moiety bound to a silica surface.

The silica matrix contains unreacted silanols on the silica surface, however their presence does not appear to contribute to the separation process (Technicol 1992). The β-CD was shown to have the widest applicability for the separation of enantiomers and achiral compounds in pharmaceutical, chemical and environmental areas and is applied in this work.

4.1.2 Basis of Stereochemical Interaction with CD

Dagleish (1952) postulated that for a chiral resolution to take place a three-point interaction is necessary. Only one of the two enantiomers is able to interact with all

three possible interaction points of the chiral stationary phase (chiral selector), where at least one of the interactions must be stereochemically based. This results in the preferential interaction of the one enantiomer with the stationary phase and thus physical separation of the two enantiomers is possible. Moreover, the interaction forces between analyte and chiral selector can also be repulsive as well as attractive (Davankov and Kurganov 1983), as long as the total sum of all interactions have a difference of a few calories in order to enable enantiomeric separation (Boehm et al. 1988).

Cyclodextrins can be used in two different ways to resolve racemic solutes: a) by using a chemically bonded CD stationary phase (Tanaka et al. 1986, Armstrong et al. 1985 and Matchett 1995) and b) by using a CD as a mobile phase additive (Matchett 1995 and Zukowsky et al. 1986). The separation mechanism in the RP mode (using buffer solutions with organic solvent) was mainly based on the formation of inclusion complexes, in which the non-polar part of an analyte enters the relatively hydrophobic cavity of the CD (Armstrong 1984 and Armstrong et al. 1985). For chiral resolution to occur it is important that there is a tight fit of the hydrophobic moiety of the analyte in the CD cavity (Armstrong et al. 1985) so that the motion of the analyte is restricted and the differential interaction with the mouth of the cavity will cause a difference in enantiomeric association constants.

Moreover, it is possible to separate chiral compounds on CDs using a polar organic mobile phase which consists of MeCN as the main solvent with MeOH as a secondary modifer to adjust retention. Additionally, glacial acetic acid and triethylamine are added to influence selectivity. The formation of a conventional inclusion complex is unlikely to occur, since the organic solvent occupies the cavity. Zukowski et al. (1992) therefore proposed enantioselectivity to be caused by hydrogen bonding between the analyte and the hydroxyl groups at the mouth of the cyclodextrin. Armstong et al. (1992) investigated the chiral resolution of β-blockers systematically and proposed a novel mechanism, where the analyte binds

to the CD torous 'like a lid' via hydrogen bonding. This mechanism was corroborated by the work of Zukowski et al. (1993) and Chang et al. (1993).

Macaudiére et al. (1987) were the first to report the use of β-CD stationary phase in the normal-phase (NP) mode using CO_2 as mobile phase which was modified with MeOH. They compared the results with the NP separation using liquid chromotography, in which no inclusion complex is formed due to the occupation of the CD cavity with hydrophobic solvent such as hexane or chloroform. In the NP mode the retention mechanism on a CD column is comparable to a diol column (Technicol 1992) and the separation of structural isomers have been mainly reported (Chang et al. 1986 and Stalcup et al. 1990). Although Armstrong and Li (1987 cited in Technicol 1992) achieved chiral separation in the NP mode, the enantioselectivity was relatively poor and this mode is either recommended for structural isomers or the use of modified β-CD was recommended for this mode. However, Macaudiére et al. (1987) achieved the enantiomeric separation of racemic amides and phosphine oxides on a β-CD using SFC and LC. As not all phosphine oxides were resolved using LC and the elution order was somewhat different, they concluded that inclusion into the cavity occurs in SFC, causing the compounds to be chirally resolved. This can only occur when assuming that the CO_2 is easily replaced from the CD cavity as otherwise no inclusion complex could occur. The resolutions obtained in SFC were further compared to those in the RP mode, however no resolution could be achieved, which led to the conclusion that the CO_2-MeOH mobile phase decreased the free volume of the β-CD cavity as a tight fit was necessary to achieve the separation.

4.1.3 Enantiomeric Separation in SFC

The properties of SCFs are beneficial to chiral separation, since a) low temperature can be applied in SFC, which enhances the interaction between analyte and the stationary phase b) lower diffusion coefficient of analytes in SCF

and lower viscosity of the mobile phase enables faster and better resolution compared to LC. Smith et al. (1995) investigated the influence of temperature on enantioselectivity in SFC and LC using potassium channel activator analogues. The compounds exhibited quite different temperature behaviour both in LC and SFC. One of the compounds, in which a n-pentanoyl group was substituted by a benzyl group was better resolved at higher temperatures (up to 42°C) in LC and SFC, which is contrary to the normal behaviour expected. The resolution of this compound may not have involved hydrogen bonding which is known to be extremely temperature dependent, but on the less temperature dependent π-π interactions (Smith et al. 1995).

The first SFC and SubFC separation was conducted by the group of Mourier et al. (1985), which investigated the resolution of phospine oxide enantiomers on a classical Pirkle's phase, having (R)-N-(3,5-dinitrobenzoyl)phenylglycine as chiral selector bonded to aminopropyl silica gel. An improvement in chiral recognition was observed when small amounts of water were added to the modifier, which was suggested was due to the deactivation of residual silanol, decreasing the adsorption energy of the silanol and thus minimising nonspecific polar adsorption (Mourier et al. 1985).

Macaudiére et al. (1989a, 1989b) and Petersson and Markides (1994) reviewed chiral separation using SFC and the latter emphasised the advantages of SFC. Additionally, since a large number of chiral separations require the use of hexane as the main solvent, a method transfer using SFC should be feasible, so that expenses in solvent purchase and solvent disposal can be saved.

4.2 Chiral Separation on β-cyclodextrin

There have only been a few applications of β-CD columns using SFC, since the mechanism in SFC appears to be based on an inclusion complex (Macaudiére et al. 1987), which can also be observed in the RP-mode in LC.

Siret et al. (1992) investigated a series of β-blockers using two chiral stationary phases derived from 3,5-dinitrobenzoyl tyrosine. The influence of solute structure, temperature, mobile phase compositions and pressure was investigated. The specific interaction of CO_2 and the analytes in this study were studied by NMR, resulting in the proposal of an in situ complexation of CO_2 and the β-blockers. The complexation was considered essential for the chiral discrimination to occur, which was corroborated by comparing the results with LC. This was further supported by the successful application of the reciprocality concept by Bargmann-Leyder et al. (1994) and the use of molecular modelling to investigate the chiral recognition mechanism (Bargmann-Leyder 1995).

Kot et al. (1994) investigated the enantiomeric resolution of β-blockers on 5 different phases, namely two Pirkle phases, two helical polymer phases and a β-CD column. They observed baseline separation for propanolol on Chiracel OD and Chiralpak AD and minor resolution on the β-CD column (Rs= 0.7). Steuer et al. (1988) used the formation of diastereomeric ion pairs to achieve the separation of chiral 1,2-aminoalcohols, whereby good enantioselectivity was obtained with the chiral selector N-benzoxycarbonylglycine-L-proline as counter-ion on a cyano column.

There has been surprisingly limited interest in the investigation of applications of β-CD columns in SFC, even though good resolution of enantiomers is achieved in this mode in LC (Technicol 1992). Nevertheless, the enantiomeric selectivity of a β-CD was tested for the resolution of optically active phenethylamines, propanolol and clofibrate analogues. These compounds have been extensively used

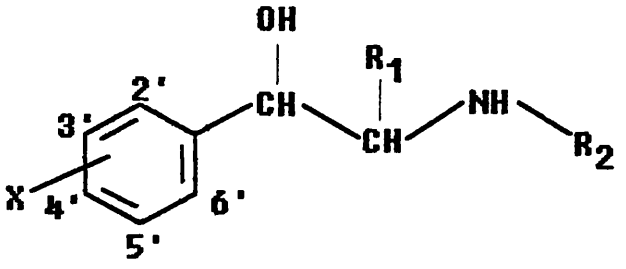
in the research group of Jefferies, which makes it possible to compare the results with those achieved using HPLC.

4.2.1 Separation of Phenethylamines

As structurally related phenethylamines are able to display different isomeric potency, it is of great interest to separate the enantiomers of these compounds (Ariens 1989).

A series of nine structurally related compounds, as indicated in Figure 4.2, were selected to investigate the enantioselective behaviour of the β-CD in SFC and to ascertain if the column offers a different enantioselectivity in RPLC and SFC.

Figure 4.2 Structure of Phenethylamines.



Compound	Name	X	R_1	R_2
1	Ephedrine	H	CH_3	CH_3
2	Oxedrine	4'-OH	H	CH_3
3	Oxilofrine	4'-OH	CH_3	CH_3
4	Norfenefrine	3'-OH	H	H
5	Etilefrine	3'-OH	H	CH_2CH_3
6	Orciprenaline	3',5'-diOH	H	$CH(CH_3)_2$
7	Noradrenaline	3',4'-diOH	H	H
8	Isoprenaline	3',4'-diOH	H	$CH(CH_3)_2$
9	Salbutamol	3'-CH_2OH, 4'-OH	H	$C(CH_3)_3$

There was no chiral resolution observed for any of the nine phenethylamines using SFC and noradrenaline did not elute at all under the applied conditions in Table 4.1.

Norfenefrine eluted also as a broad, tailing peak, however it showed a markedly better peak shape than isoprenaline. The reason for this behaviour can be related to the structure of the phenethylamines. Norfenefrine possesses a primary amine group and was able to interact strongly with existing silanol groups or is ionised at the conditions used. This was unexpected as the addition of 1% of iso-PA should have been sufficient to deactivate the residual silanols and/or to suppress ionisation, which was achieved when analysing the alkaloids. In the case of the alkaloids, the amino group is however sterically hindered, enabling the elution of the more basic alkaloids (pK_a~11) compared to the phenethylamines (pK_a~9.5). Phenethylamines were separated by Berger and Wilson (1993b) on a non-chiral diol stationary phase, and they observed only a slightly tailing peak for β-phenethylamine. As the β-CD column exhibited similar behaviour as the diol phase when separating the alkaloids, the same was expected for the phenethylamine analogues. Since the only difference between the phenethylamine used by Berger and Wilson (1993b) and norfenefrine was the presence of two additional hydroxy groups, these must have caused the tailing and indicated solubility limitations. The comparison of ephedrine and oxilofrine, having an additional hydroxyl group on the aromatic ring, revealed that the retention factor increased by a factor of 3.5. This hypothesis was further confirmed by the elution behaviour of isoprenaline, which showed severe tailing despite the absence of a primary amino group. Isoprenaline possesses 3 hydroxy groups and these must be responsible for the severe tailing. It is therefore not surprising that noradrenaline with 3 hydroxy groups and a primary amino group could not be eluted.

Table 4.1 Retention factor of phenethylamines on a β-CD column.
Conditions: 25°C, 15%MeOH + 1.0% iso-PA, 200kg/cm² and a flow rate of 0.7ml/min.

Compound	Retention factor
Ephedrine	2.14
Oxedrine	7.95
Oxilofrine	7.17
Norfenefrine	~10, tailing peak
Etilefrine	5.10
Orciprenaline	9.22
Noradrenaline	no elution <30min
Isoprenaline	~10, extremely tailing "peak"
Salbutamol	7.20

When comparing isoprenaline and orciprenaline, the orciprenaline was eluted with a k~9 without significant tailing, indicating that the position of the hydroxygroups also influenced the solubility markedly.

When these results were compared to the systematic study of Matchett (1996), in which chiral separation was investigated using the RP and polar organic mode, the retention mechanism appeared to be different in all three modes. In the RP mode the phenethylamine eluted with retention factors between 2-4.5 and chiral resolution was observed for ephedrine (36% CRF), oxilofrine (67% CRF) and orciprenaline (39%), whereas the retention factors in the polar organic mode were considerably higher (k~40->100) and only salbutamol was partially (32% CRF) resolved. Considering that in both the RP-LC and the SFC mode an inclusion complex was proposed (Armstrong et al. 1985 and Macaudiére et al. 1987), the same enatioselectivity would have been expected. However, as already emphasised by Macaudiére et al. (1987), the free volume in a cyclodextrin ring is not a fixed entity, resulting in deviating results. In spite of proposing the inclusion complex, Macaudiére compared the NP-LC with the SFC mode, which resulted in good correlation with most of the compounds investigated. Inclusion complexes are not considered to take place in NP-LC, making the assumption of inclusion

complexes to exist in SFC doubtful. Since the results obtained in the polar organic mode did not correspond to the SFC results either, it can be concluded that the separation mechanism of chiral compounds must be unique and hence offers an additional way of chirally resolving enatiomers in SFC.

4.2.2 Propanolol and Clofibrate Analogues

Propanolol [1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol] is a widely applied β-blocker in the treatment of various cardiovascular disorders. It is known, that the R and S-enantiomers of propanolol exhibit different pharmacological effects *in vivo* (Krstulovic 1989) and it was determined that the L-enantiomer is 100 times more potent than the D-enantiomer (Wainer 1989).

Numerous separations of propanolol have been achieved using various chiral columns in SFC. The following investigation studies the optimisation of parameters in SFC in order to chirally resolve the structurally related compounds based on the drug propanolol. The structure can be seen in Figure 4.3, where four of the six compounds were the naphthyl derivative (compounds 1, 2, 3 and 4) and two were the phenyl derivatives (compounds 5 and 6). Each of these compounds had either an i-propyl or t-butyl group on the secondary amine, except for compound 4, which had a CONH-ethyl group. These analogues were manufactured by Zeneca Pharmaceutical with the intention of producing additional β-blockers. For the *in vivo* tests it is esssential to test the individual enantiomers to see whether potential β-blocker have been produced, therefore it is necessary to separate the individual enantiomers to make them available for the tests.

Figure 4.3 Structures of propanolol analogues 1-6.

$$R_1 - O - CH_2 - CHOH - CH_2 - NH - R_2$$

P1, R_1 = naphthyl, R_2 = *i*-propyl

P2, R_1 = naphthyl, R_2 = *t*-butyl

P3, R_1 = naphthyl, R_2 = ethyl

P4, R_1 = naphthyl, R_2 = CONH-ethyl

P5, R_1 = phenyl, R_2 = *i*-propyl

P6, R_1 = phenyl, R_2 = *t*-butyl

The initial investigation using the following conditions: 25°C, 15% MeOH + 1.0% iso-PA, 200kg/cm^2 and a flow rate of 0.7ml/min, resulted in the partial resolution of compound 5 and 6 which can be seen in Figure 4.4.

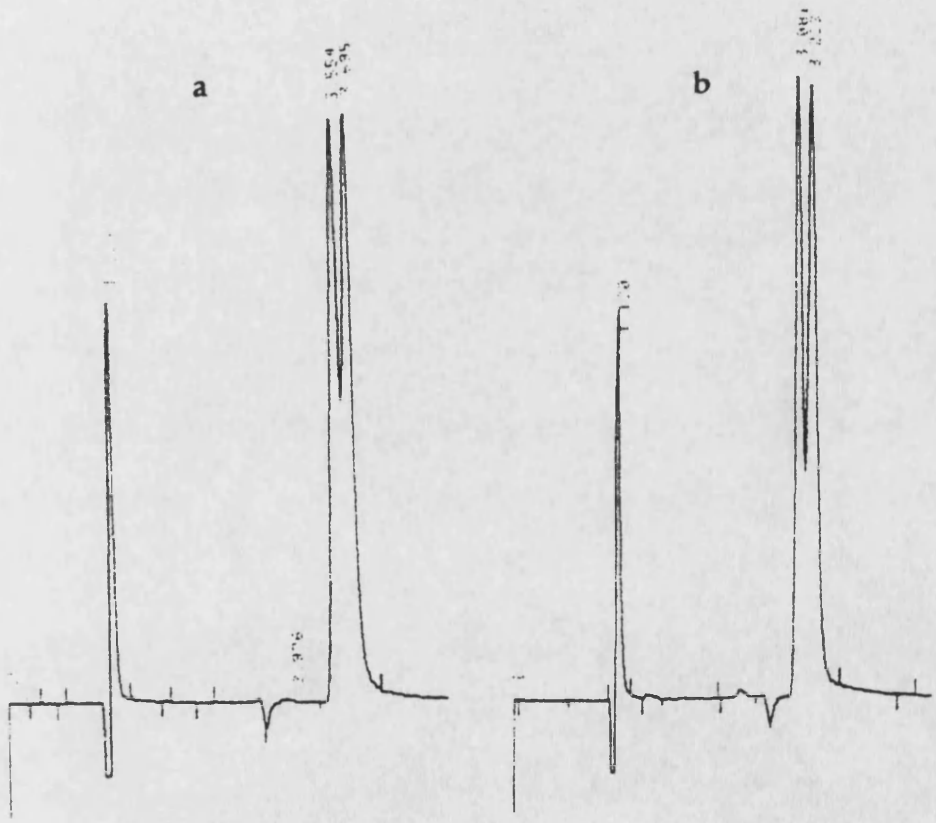


Figure 4.4 Chiral resolution of a) P5 and b) P6 using a β-CD column.
Conditions: 25°C, 15%MeOH + 1.0% iso-PA, 200kg/cm^2, 0.7ml/min.

The results from the separation of the propanolol analogues are given in Table 4.2 and these enable the comparison to be made with the results obtained by Matchett (1996) using RP and the polar organic mode in LC. Selectivity in this chapter refers to enantioselectivity, which is defined as the ratio of the retention factors of the two enantiomers.

Table 4.2 Retention factor and enantio-selectivity obtained from the chiral resolution of propanolol analogues on β-CD[a]

Compound	Retention factor k_1	Retention factor k_2	Selectivity
P1	2.717		
P2	2.225		
P3	3.399		
P4	2.716		
P5	2.185	2.311	1.058
P6	1.751	1.878	1.072

[a] Conditions: 25°C, 15% MeOH + 1.0% iso-PA, 200kg/cm^2, 0.7ml/min.

The elution order was according to the results in Table 4.2 **P6 < P5 < P2 < P4 ~ P1 < P3.** It is surprising that the early eluting compounds are resolved, as one would expect them to interact the least with the cyclodextrin moiety and therefore to be the least resolved. For chiral resolution to occur the strength of interaction is not the determining factor, however the type of interaction is, where at least one must be chirally dependent. The same phenomena was observed by Stalcup et al. (1991).

Matchett (1996) achieved chiral resolution for compound P1 (5% CRF), P2 (34% CRF) and P5 (30% CRF) in the RP mode when the MeOH concentration was lowered to 5% with the elution order of **P6 < P5 < P3 ~P1 ~P2 << P4.** The elution order was surprisingly similar to that obtained in SFC, considering the different solubility behaviour of an aqueous solution and the SC-CO_2, which is regarded as a NP mode.

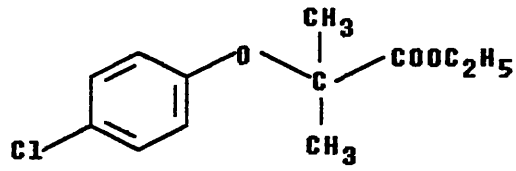
In the polar organic mode in LC, it was possible to separate all the analogues except compound 4, which was only slightly retained (Matchett 1996). The elution order was **P2 (89%CRF) < P5 (93%CRF) < P1 (62%CRF) < P6 (78%CRF) < P3 (17%CRF)**, which deviated also significantly from that in SFC and therefore indicated that the presence of a lid-rim complex as an alternative to the inclusion complex might be less probable. However, in order to allow a valid comparison, the addition of glacial acetic acid would be recommended.

In the work of Matchett (1996) the use of CDs as mobile phase additives was investigated on either a RP column or a porous graphitic column PGC. Using the PGC column and a derivatised peractyl- β-CD the elution order was **P6 < P5 < P3 < P1 < P2 << P4** and compound P5 and P6 were chirally resolved, with 47% CRF and 42% CRF, respectively. The elution order does not correspond to that in SFC, which was expected due to solubility differences, nevertheless, the same compounds were resolved. This may be coincidental, however it may also be indicative that the modifier or the strong additive was strongly adsorbed onto the ring of the cyclodextrin moiety and therefore created additional or completely different interaction sites, however there has not been any reports about different additives and modifiers changing the enantioselectivity, which would be expected in such a case.

A further optimisation of the compounds 5 and 6 was investigated in conjunction with the clofibrate analogues, since the separation was based on the same principle and can be explained concomitantly.

Figure 4.5 shows the structure of clofibrate, the ethyl ester of an aryloxyalkanoic acid, being the most widely used antihyperlipidemic agent (Wolff 1979).

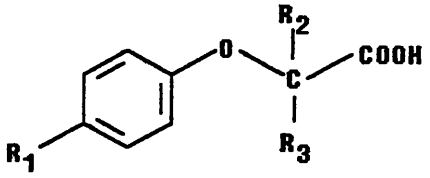
Figure 4.5 Structure of Clofibrate



Despite extensive clinical investigations, the mode of action of clofibrate is still not completely understood, however its main effect lies in the reduction of the endogenous triglycerides of the very low density lipoprotein (Wolff 1979).

A series of clofibrate analogues, ZP1-ZP9 and 3 similar compounds ZP10-ZP12 (Figure 4.6) were investigated to find potential successful compounds for the separation on the β-CD column.

Figure 4.6 Structure of clofibrate analogues



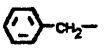
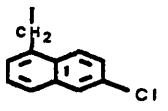
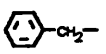
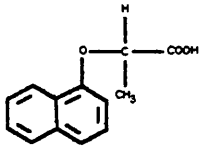
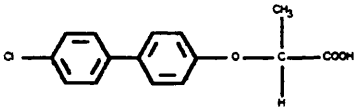
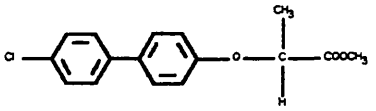
Compound	R_1	R_2	R_3
C1	H	CH_3	H
C2	H	CH_3CH_2	H
C3	Cl	CH_3	H
C4	Cl	CH_3CH_2	H
C5	Cl	CH_3	CH_3CH_2
C6	Cl	CH_3	
C7	Cl	CH_3	
C8	Cl	CH_3	
C9	Cl	$(CH_3)CH$	
C10			
C11			
C12			

Table 4.3 gives the chromatographic parameters for all compounds and as seen from the Table the compounds C7, C8 and C11 were chirally resolved on the β-CD column.

Table 4.3 Chromatographic and chiral parameters obtained from the separation of the clofibrate analogues

Compound	Retention factor k_1	Retention factor k_2	Selectivity
C1	3.537	—	—
C2	3.267	—	—
C3	3.933	—	—
C4	3.626	—	—
C5	3.392	—	—
C6	6.911	—	—
C7	8.152	8.918	1.094
C8	7.596	8.213	1.081
C9	3.934	—	—
C10	4.870	—	—
C11	6.812	7.030	1.032
C12	7.954	—	—

* Conditions: 25°C, 15% MeOH + 1.0% iso-PA, 200kg/cm^2 and a flow rate of 0.7ml/min.

Compound C7 with the naphthyl group in position R_3 had the longest retention time and comparing it with the compound C3, whose R_3 position is occupied by a hydrogen, it showed a significant difference in retention factor to that of compound C3, leading to the conclusion that a tight inclusion complex was formed with the naphthyl moiety and this allowed the chiral recognition to occur. The chiral enantioselectivity achieved with C8, involved either the inclusion of the pyridinyl or phenyl residue, however it cannot be deduced from the data which of the two aromatic moieties in C8 took part in the inclusion complex.

The comparison of C11 and C12 led to the conclusion, that the presence of the free carbonyl acid was important for the chiral recognition to occur since the ester (C12) was not resolved under the same conditions.

Before investigating all the physical parameters and their extent on influencing the chiral recognition mechanism, the influence of the presence of the amine on the separation of the acids C1-C12 was investigated. In the presence of the iso-PA it

was possible to elute C7 and C8 at around 10 minutes and in both cases a baseline separation was achieved. Contrarily, 30% MeOH was necessary to achieve the elution of C7 (19 min) and C8 (22 min), however the chiral resolution deteriorated markedly. These results are very interesting, since this indicated that the iso-PA either took part in the chiral recognition process or improved solubility to such an extent that firstly the peak width decreased and secondly the efficiency of the separation increased due to the smaller amount of modifier necessary.

For the following study, the propanolol analogues were included in order to cut down on additional graphs and it may also be interesting to compare the behaviour of amines and acids.

Influence of the amine concentration: The amount of iso-PA in MeOH was varied in order to establish the optimum levels of iso-PA and also to study the influence of the amine concentration on the chiral recognition mechanism. As seen from Figure 4.6 only the retention factor of the clofibrate analogues (C7, C8 and C11) decreased with increasing amine content.

It was assumed, that the mechanism was a NP ion-pair mechanism, since the retention decreased with increasing amine concentration. In RP ion-pair chromatography retention increases with increasing concentration of counter ion (Johansson et al. 1978 and Steuer et al. 1988). The formation of an ion-pair with the clofibrate analogues must have resulted in the formation of a more soluble ion pair and hence shorter retention on the column. This behaviour was reported by Pettersson (1989) for the (R/S)-alprenolol using N-benzoxycarbonylglycyl-L-proline ZPG as counter-ion on a diol stationary phase.

Another possibility could be the increase of solvent power when adding the amine to the fluid, the deactivation of silanols, preventing non-specific interaction of the solute, or the competitive adsorption of amine onto the hydroxyl groups on the rim of the cyclodextrin cavity. The latter should also have affected the

propanolol analogues P5 and P6, however as seen from the Figure the retention of the propanolols remained constant.

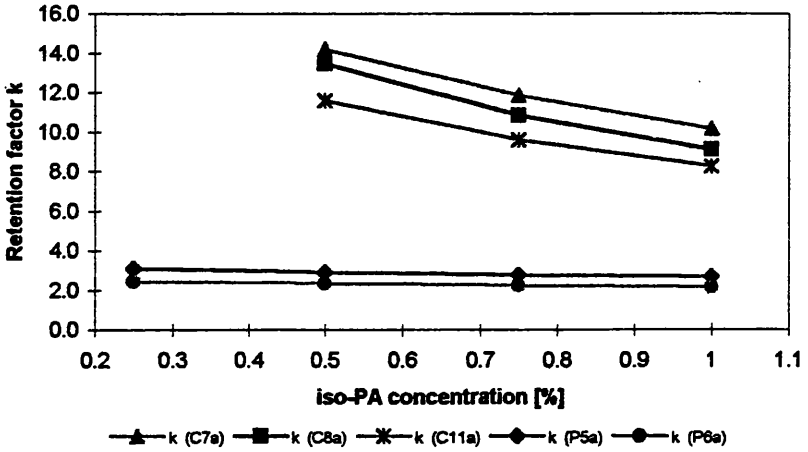


Figure 4.6 Influence of iso-PA concentration on the retention factor.
Conditions: β-CD, 25°C, 15%MeOH + 1.0% iso-PA, 200kg/cm^2, 0.7ml/min.

Figure 4.7 depicts the resolution of the compounds and only the chiral resolution of C11 appeared to be significantly influenced by the amine concentration, indicating that the added amine had an additional function than that of increasing the solubility as this would have affected both enantiomers to the same extent. The evaluation of the resolution by means of baseline width suffered the same drawback as mentioned in the separation of the alkaloids and the resolutions are overestimated approximately by a factor of 1.3.

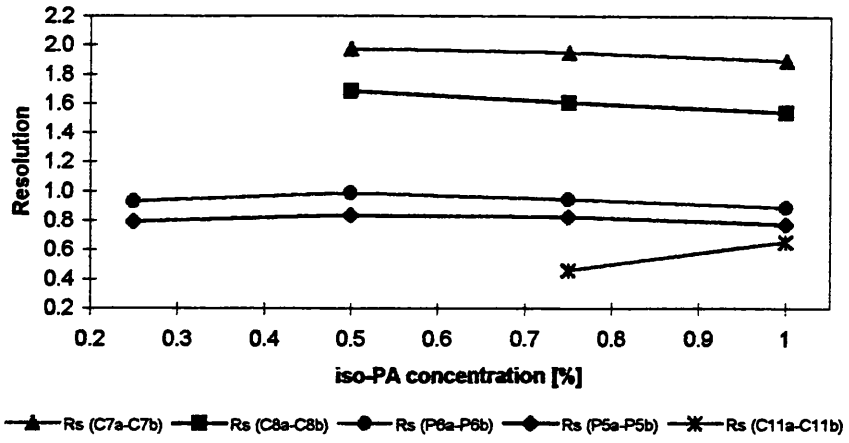


Figure 4.7 Variation of resolution of clofibrate and propanolol analogues with amine concentration.
Conditions: as in Figure 4.5.

The selectivity of the C11 with the concentration of the iso-PA, given further indication of a different mechanism compared to the remaining compounds.

Temperature: The influence of temperature was investigated at constant pressure and density, in order to highlight the differences. The investigation at constant density will show both the effect of increasing the vapour pressure and decreasing interaction of the solute with the stationary phase with increasing temperature. The influence of decreasing solubility of the individual compounds with temperature will not be relevant as the solvent power of the fluid is maintained. In contrast, the study at constant pressure will highlight the above two factors in addition to decreasing solubility of the compounds due to decreasing density when temperature is increased at constant pressure.

The retention factor remained almost constant with increasing temperature (22 - 30°C) at constant pressure, as an increase in volatility or decrease in the strength of interaction must have been counteracted by the decrease in solvent power of the fluid which can be seen in Figure 4.8. The same investigation at constant density resulted in a decrease in retention time (Figure 4.8) with increasing temperature and corresponds to the behaviour observed by Siret et al. (1992) for oxeprenolol (β-blocker).

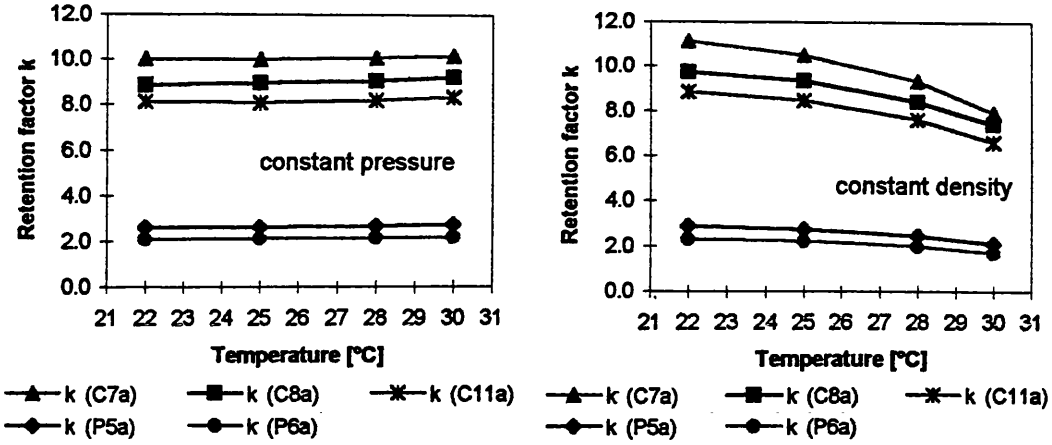


Figure 4.8 Selectivity change with increasing temperature
Conditions: for constant pressure 150 kg/cm^2, 15% MeOH + 1.0% iso-PA, 0.7ml/min, for constant density Table 4.5, 15% MeOH + 1.0% iso-PA, 0.7ml/min.

The pressures necessary to maintain the density during temperature change were calculated using the SF Solver and are listed in Table 4.4.

Table 4.4 Required pressure at different temperature to maintain a density of 0.946 g/ml

Temperature [°C]	Pressure [kg/cm^2]
22	123
25	138
28	169
30	215

The evaluation of the retention factor to produce van't Hoff plots (log k vs 1/T °K) resulted in low R^2 values for the linear regression of log k versus 1/T (0.91) at constant density. The gradient, which according to Smith et al. (1995) and many other authors corresponds to the enthalpy of transfer, was shown by Roth (1991) to include several thermodynamic quantities and does not represent purely the enthalpy of transfer. The enthalpy was calculated for P5 to be 1.44kJ/mol which is close to what Smith et al. (1995) obtained for one of the compounds. Since all the compounds (clofibrate and propanolol analogues) had the same gradients when calculated in the plot ln k versus 1/T, it can be concluded that the same

enantioselective recognition mechanism in all the compounds prevailed. Smith et al. (1995) found deviating behaviour between the investigated compounds, one of which gave better resolution with increasing temperature. It was concluded that in this case the enantioselective recognition mechanism was primarily based on the π-π interaction (Smith et al. 1995). Even though no π-π interaction were present using the β-CD column, the interactions showed only minor temperature dependence, which might suggest that an inclusion complex is present.

Siret et al. (1992) further evaluated the enthalpy difference in affinity of both enantiomers for the CSP by plotting ln α versus 1/T. The group calculated a value of 5.2 kJ/mol for oxeprenolol, however in this study only 0.15 kJ/mol was observed for the enthalpy difference between the enantiomers for compound P5. This indicated that the enantioselectivity of the oxeprenolol was far more dependent on temperature than P5.

The plots of resolution versus temperature at constant pressure and density showed linearly decreasing resolution with increasing temperature, which was expected as the interaction between the analyte and CSP decrease with increasing temperature.

The same behaviour can be observed for the selectivity change at constant pressure and density for all investigated compounds as seen in Figure 4.9. The unexpected small changes of selectivity with temperature in Figure 4.9 must be caused by the negligible influence of temperature on the interaction between analyte and stationary phase, suggesting that the chiral recognition mechanism is not solely based on hydrogen bonding. The other possibility is, that the inclusion complex in the cyclodextrin cavity was not influenced markedly by temperature.

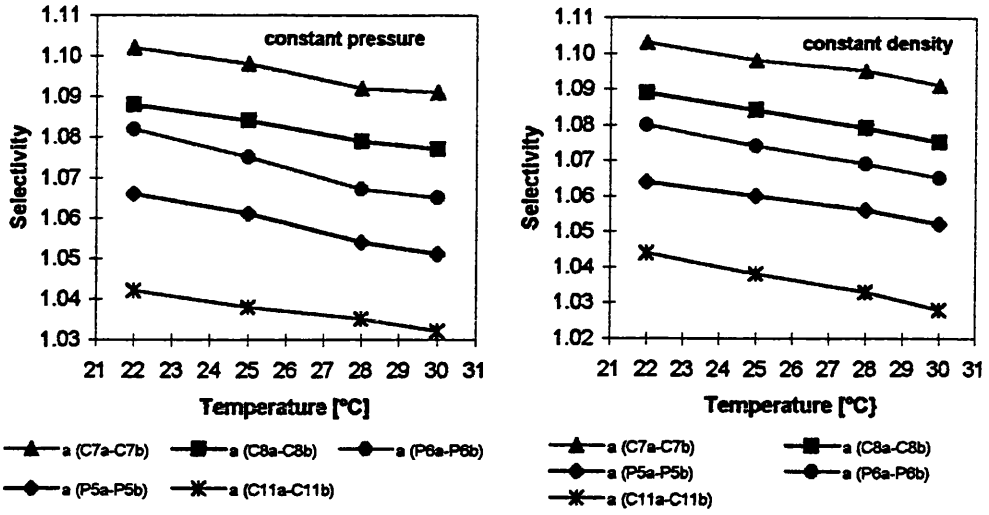


Figure 4.8 Selectivity change with increasing temperature.
Conditions: for constant pressure 150 kg/cm², 15% MeOH + 1.0% iso-PA, 0.7ml/min flow rate, for constant density Table 4.5, 15% MeOH + 1.0% iso-PA, 0.7ml/min flow rate..

No comparison with RP-LC was possible since Matchett (1996) did not report selectivity changes. In contrast, the investigation by Siret et al. (1992) selectivity changed to a relatively greater extent with temperature despite of the recognition mechanism being based on π-π interactions, which were considered in the study of Smith et al. (1995) to be less temperature dependent.

Pressure: Increasing pressure decreased the retention factor as would be expected due to the increasing solvent power of the fluid. The resolution shows a more interesting pattern, as the resolution for the propanolol analogues increased initially, which must be due to better solubility and hence improved efficiency, but then decreased at pressures higher than 150kg/cm². The most likely explanation for this was the increase in density with increasing pressure, thus decreasing the diffusion coefficient and in turn the efficiency. As seen from Figure 4.10 the resolution of the clofibrate derivatives show a continual decrease since the increase in pressure caused an decrease in diffusion coefficient and thus a less efficient separation.

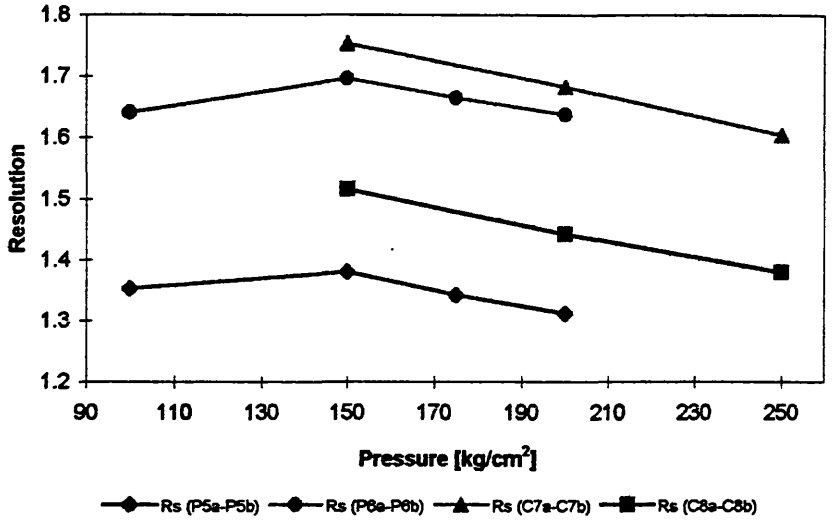


Figure 4.10 Influence of pressure on resolution of propanolol and clofibrate analogues.
Conditions: for P5, P6: β-CD, 25°C, 7.5% MeOH + 1.0% iso-PA, 0.7ml/min and for C7, C8: β-CD, 28°C, 18% MeOH + 0.5% iso-PA, 0.7ml/min.

The selectivity of the propanolol analogues shown in Figure 4.11 experienced a continuous increase in selectivity with increasing pressure, highlighting the decreasing efficiency shown in Figure 4.10 with increasing pressure above 150 kg/cm².

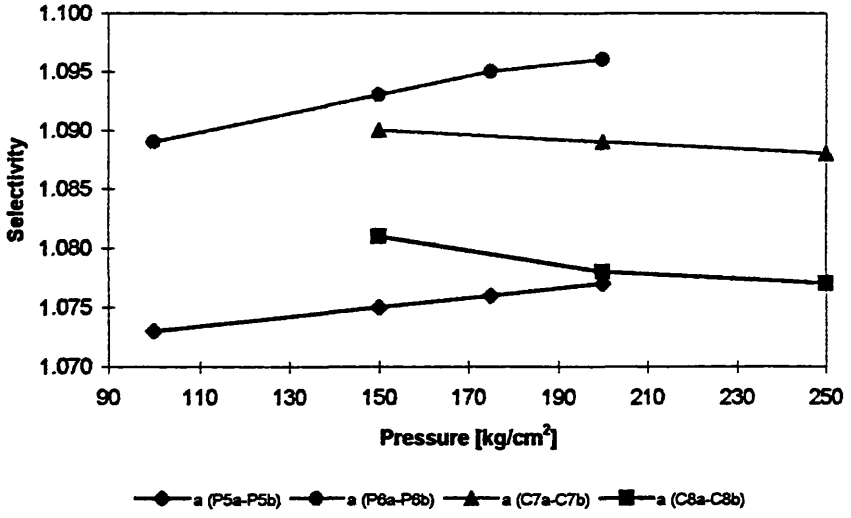


Figure 4.9 Influence of pressure on selectivity of propanolol and clofibrate analogues
Conditions: for P5, P6: β-CD, 25°C, 7.5% MeOH + 1.0% iso-PA, 0.7ml/min and for C7, C8: β-CD, 28°C, 18% MeOH + 0.5% iso-PA, 0.7ml/min.

The comparison of resolution and selectivity is of interest, as it highlighted the influence of diffusion and thus the efficiency of a separation. The clofibrate analogues showed a slower decrease in selectivity in Figure 4.11 with pressure than the resolution in Figure 4.10, indicating the loss in efficiency.

Modifier concentration: Retention factors of all the compounds decreased with increasing MeOH concentration due to the increase in solubility. This corresponded to the results in the non-chiral separations.

The resolution of the enantiomeric separation decreased with increasing MeOH concentration, which was also observed by Kot et al. (1994). Additionally, this group observed that the selectivity underwent only minor changes with modifier concentration. The same was observed in this study and the influence of modifier concentration on resolution and selectivity can be seen in Figure 4.12.

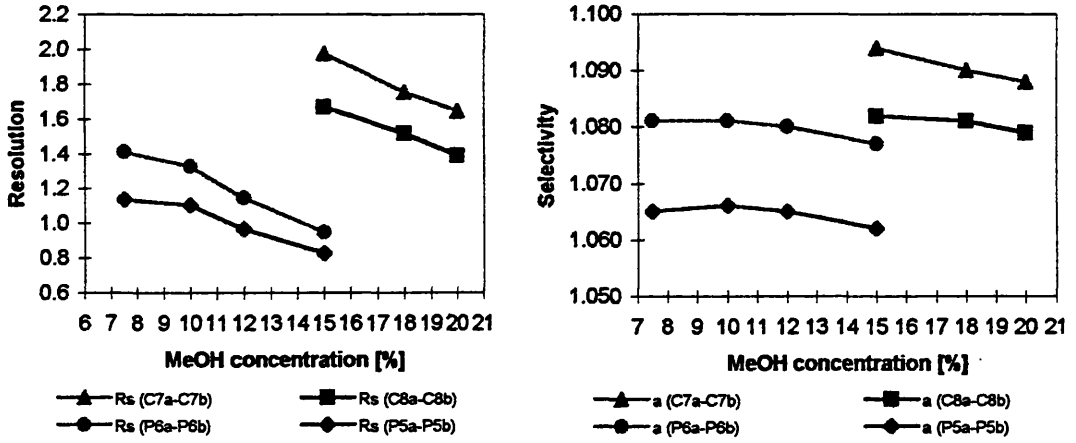


Figure 4.12 Variation on resolution and selectivity with increasing Modifier concentration.
Conditions: β-CD, 25°C, MeOH + 0.75% iso-PA, 150 kg/cm^2, 0.7ml/min.

The reason for the decreasing resolution with increasing modifier concentration was a result of the decreasing diffusion coefficient and thus decreasing efficiency.

4.3 Chiral Separation on (S)-NEC-β-CD

Armstrong et al. (1990b) developed a range of derivatised cyclodextrins for NP chromatography in order to extend their applicability. Around 50 chiral stationary phases are available today, however many of these show overlapping enantioselectivities, so that almost 90% of the chiral compounds are separated by 6 or 7 different stationary phases (Armstrong et al. 1991a). However, there is still a need for additional CSPs.

The (S)-NEC-β-CD is produced by derivatising β-CD with (S)-(+)-1-(1-naphthyl)ethylisocyanate at a large reagent excess to derivatise some of the 21 hydroxyl groups on the β-CD, resulting in a carbamate linkage (Armstrong et al. 1990). The average degree of substitution are approximately 6 (S)-NEC groups, whereby the number of (S)-NEC groups influences the retention and the enantioselectivity (Armstrong et al. 1991b). The NEC group possesses more rotational freedom compared to the cyclodextrin, and are considered to be an important moiety in chiral recognition mechanism, since π-π interaction can take place on the aromatic naphthyl and the carbonyl substituents. Moreover, the residual hydroxyl groups of the CD and the hydrogen bonding and strong dipole-dipole interactions possible with the carbamate linkage are also attributable to the chiral recognition mechanism (Stalcup et al. 1991). It is thought that an inclusion complex does not appear to play an important role (Armstrong et al. 1991b), although in various separations it is possible that both the CD moiety and the NEC group contribute to the recognition mechanism.

NEC- β-CD is considered to be a true multimodal column since it is possible to perform separations in NP chromatography (Stalcup et al. 1991) in the RP mode (Armstrong et al. 1991b) and in the polar organic mode (Armstrong et al. 1991a) and achieve different enantioselectivity in each mode.

4.3.1 Phenethylamine

The properties of the phenethylamines were already accounted for in section 4.2.1, the structures of which can be seen in Figure 4.2. The same conditions as set out in that section were used for the separation on the (S)-NEC-β-CD. Norfenefrine and isoprenaline eluted again with a very broad, tailing peak and no elution was observed for noradrenaline. In general, the elution order of the phenethylamines on the (S)-NEC-β-CD was the same as on the β-CD, however they were retained to a greater extent, but no chiral resolution was observed.

4.3.2 Propanolol and Clofibrate Analogues

General information about propanolol and clofibrate analogues were presented in section 4.2.2, the structures of the compounds can be seen in Figures 4.3 and 4.5 respectively. Table 4.5 lists the results obtained from an initial investigation and shows that the elution order was almost identical to that on β-CD, however P2 was eluted earlier than compound P5 which might indicate that the interaction of P2 and the stationary phase was hindered due to presence of the large naphthylethyl moieties.

Table 4.5 Retention factor and enantio-selectivity obtained from the chiral resolution of propanolol analogues on (S)-NEC-β-CD[a]

Compound	Retention factor k_1	Retention factor k_2	Selectivity
P1	5.851		
P2	4.59		
P3	7.338		
P4	5.152		
P5	4.815	4.910	1.020
P6	3.692	3.852	1.043

[a]Conditions: 25°C, 15% MeOH + 1.0% iso-PA, 200kg/cm^2, flow rate of 0.7ml/min.

Compound P5 and P6 were only slightly resolved on (S)-NEC-β-CD , although compared to the separation on the β-CD the separation was markedly worse. Figure 4.13 shows the separation of the propanolol analogues on the (S)-NEC-β-CD column as comparison to the separation on the β-CD column (Figure 4.4).

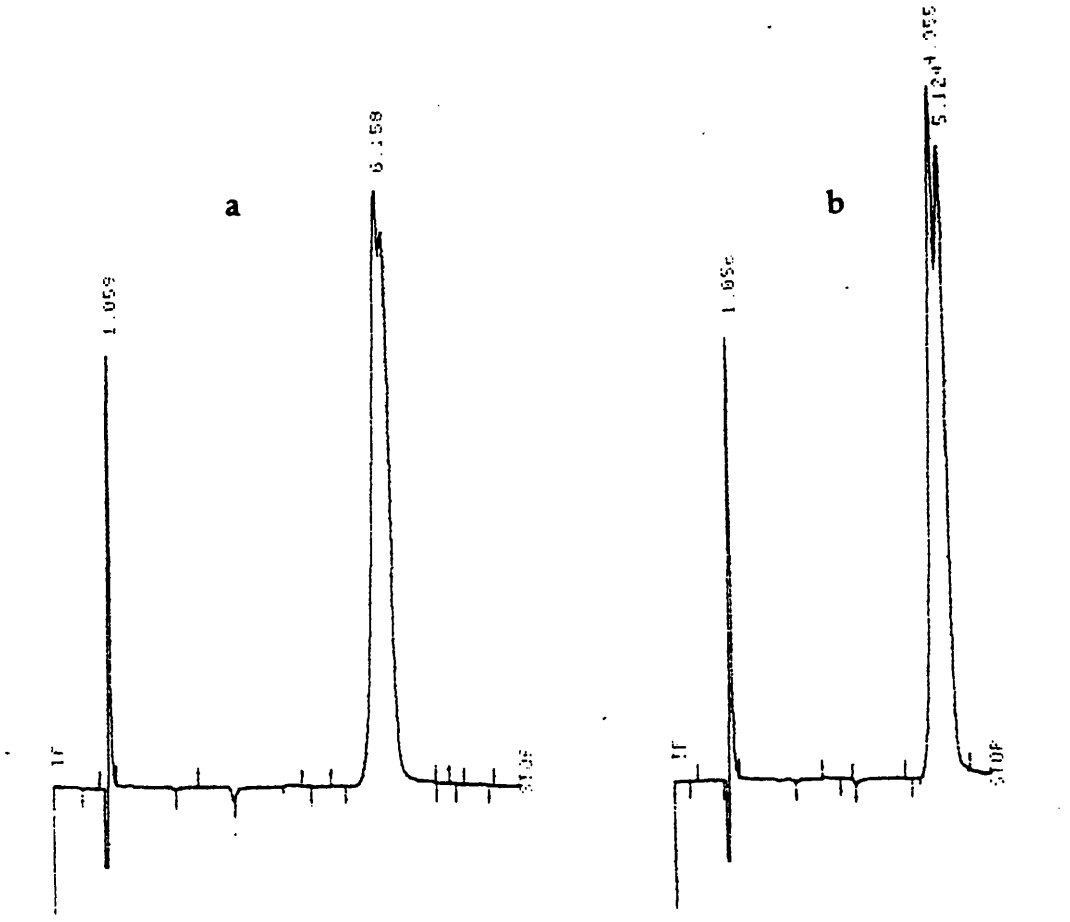


Figure 4.13 Chiral resolution of compounds a) P5 and b) P6 using a (S)-NEC-β-CD column.
Conditions: 25°C, 15% MeOH + 1.0% iso-PA, 200kg/cm^2, 0.7ml/min.

Since the resolution was poor and a major improvement by changing physical parameters was not anticipated, no further investigation was conducted on the propanolol analogues.

The separation of the clofibrate analogues was however far more successful on the (S)-NEC-β-CD column than on the β-CD column, as 9 of the 12 investigated compounds could be chirally resolved with superior enantioselectivity, suggesting

that the naphthylethylcarbamate moiety had a major influence on the enantioselectivity. Table 4.7 shows the results of the separation and lists further the selectivity on the β-CD column.

Additionally, the comparison of the elution order between the two columns revealed that the compounds were eluted in almost the same order, except that C9 elutes before C3 on the (S)-NEC-β-CD column. This was unexpected as it was anticipated the two columns would exhibit quite different retention behaviour. Macaudiére et al. (1987) predicted the presence of inclusion complexes in SFC with a β-CD column, however an inclusion complex was not anticipated for the (S)-NEC-β-CD column due to the evidence of earlier publications (Armstrong et al. 1991a, b). A possible explanation for the similarity of retention behaviour could be the naphthyl moiety fulfilled a similar task to the cyclodextrin cavity, it is merely used to align the compound, so that other interactions such as hydrogen bonding and dipole-dipole interaction can take place. One of these additional interaction must be stereochemically based (Dagliesh 1952) and its most likely to be the hydrogen bonding and dipole-dipole interactions. This would also explain why most of the 50 available chiral columns produce similar enantioselectivity.

Table 4.6 Chromatographic and chiral parameters obtained from the separation of the clofibrate analogues [a]

Compound	Retention factor k' [b]	Selectivity (S)-NEC-β-CD	Selectivity β-CD
C1	4.719	1.138	—
C2	4.120	1.097	—
C3	5.493	1.200	—
C4	4.823	1.186	—
C5	4.401	1.086	—
C6	11.682	—	—
C7	15.212	1.220	1.094
C8	13.258	1.136	1.081
C9	5.181	1.153	—
C10	6.523	—	—
C11	11.396	1.243	1.032
C12	0.570	—	—

[a] Conditions: 25°C, 15%MeOH + 1.0% iso-PA, 200kg/cm², 0.7ml/min.
[b] retention factor of first eluting enantiomer.

By comparing the degree of retention taking place on both columns in Figure 4.14, it can be seen that additional aromatic moieties enhanced the retention on the (S)-NEC-β-CD column quite considerably.

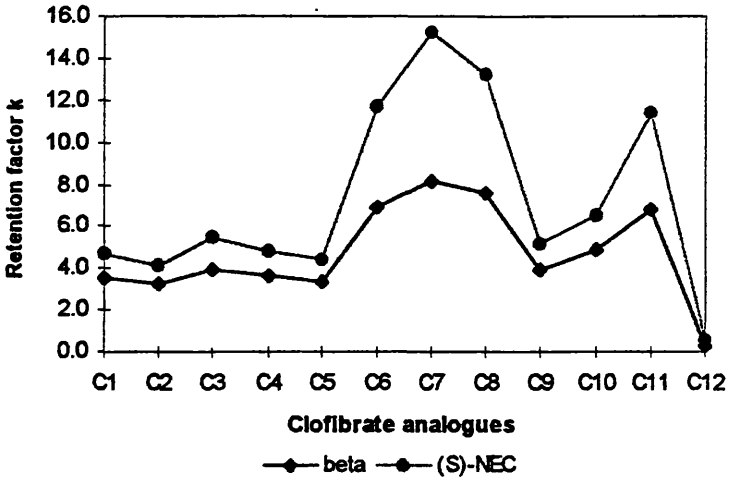


Figure 4.14 Retention factors of clofibrate analogues on (S)-NEC-β-CD and β-CD column.
Conditions: 25°C, 15%MeOH + 1.0% iso-PA, 200kg/cm^2, 0.7ml/min.

However, there were two compounds not retained to the degree as anticipated. C9 should have a similar retention enhancement on the (S)-NEC-β-CD column as C6, however the i-propyl group on C9 must have an repulsive effect on retention. As proposed by Davankov and Kurganov (1983) it is also possible for a repulsive effect to cause chiral enantioselectivity. The reason why C10 was less retained on the (S)-NEC-β-CD column must also be due to sterical hindrance since the naphthyl moiety was α-substituted and hence did not allow complete alignment.

From the initial results of the chiral resolution of analogues a conclusion can be drawn for the structural requirements of compounds to be resolved on the (S)-NEC-β-CD column. The substitution of the methyl group (C1) with an ethyl (C2) group on R_2 of C1 caused a decrease in selectivity, however, replacing the hydrogen with a chlorine on R_1 increased α (C3). The introduction of the chlorine in the aromatic ring made the phenyl moiety more π-acidic, thus increasing the

interaction force. Substituting hydrogen (C3) on R_3 with a benzyl group (C6), resulted in a complete loss in enantioselectivity. Only when the methyl group on R_2 (C6) was substituted with an i-propyl group (C9) was the enantioselectivity maintained. The introduction of a naphthylmethyl moiety on R_3 (C7) achieved the highest enantioselectivity. Even though the selectivity was reduced as the hydrogen on R_3 (C3) was substituted with pyridinyl (C8), the resolution was still sufficient. From the comparison of C11 and C12 one can conclude that the free acid was necessary for hydrogen bonding and thus for chiral recognition to occur.

In the following section only the influence of modifier was investigated in order to find the fastest elution time while still retaining a resolution of about 2, taking the overestimation by a factor of 1.3 into account. The variation in pressure to decrease retention and maintain resolution would be more advantageous, however the change in modifier was more efficient in achieving shorter elution times.

Modifier concentration: The levels of modifier were varied so that a resolution of the pairs of enantiomers was maintained. Figure 4.15 shows the variation in resolution with modifier and the level of modifier necessary to achieve the desired resolution. The resulting retention factor can then be estimated from a graph depicting retention factor versus MeOH concentration.

The formation of two groups can be seen in Figure 4.15 which behaved similarly. These were C1, C7, C8 and C9, C11 and C2, C3 and C4, C5, however when studying the structures in Figure 4.6 there was no obvious explanation for the grouping, except for C3 and C4 which are very similar.

An interesting behaviour can be seen on C8, the only compound with a nitrogen containing moiety, as the resolution of this compound did not degrade as quickly as others. However, when the efficiency of the C8 peak was calculated the plate height was 68μm compared to 50-58μm for the remaining compounds. The plate heights calculated for the (S)-NEC-β-CD column was around a third higher than that reported by Bargmann-Leyder et al. (1994) and Steuer et al. (1988). This might

be due to the lower efficiency of the CD columns in general and/or the column dimension of 2.1mm i. d. compared to 4.6mm i. d. used in the above mentioned studies. The plate height for the β-CD column was between 35-40μm, which was still only half as efficient as the columns used in other studies.

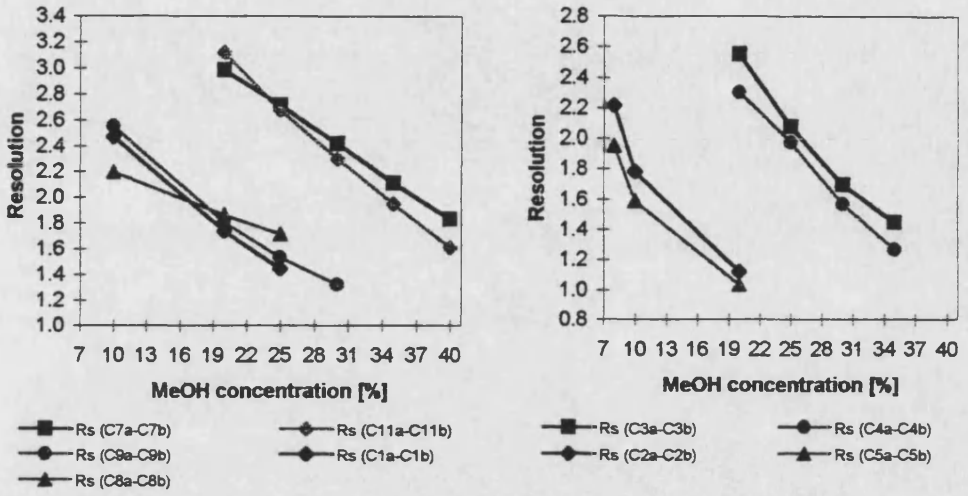


Figure 4.15 Resolution of clofibrate analogues with variation in modifier concentration on the (S)-NEC-β-CD column.
Conditions: 25°C, MeOH + 1.0% iso-PA, 200kg/cm^2, 0.7ml/min.

Figure 4.16 demonstrates the efficiency of chiral SFC and the speed with which separation can be conducted when all the parameters, including the stationary phase, are optimised.

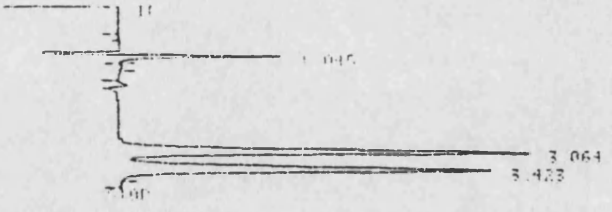


Figure 4.16 Chiral resolution of compounds C3 on a (S)-NEC-β-CD column
Conditions: 25°C, 30% MeOH + 1.0% iso-PA, 200kg/cm^2, 0.7ml/min.

4.3.2 Diastereomeric Separation of Bn-ether

CDs are widely used for the separation of enantiomers, however they are also applied for the separation of geometrical and structural isomers (Armstrong and DeMond 1984).

Bn-ether is part of the synthesis of a cyclic adenosine diphosphate, which is a calcium release agent and therefore important in cell signalling pathways. The aim was to separate 4 stereoisomers, which are shown in Figure 4.17 on the (S)-NEC-β-CD column.

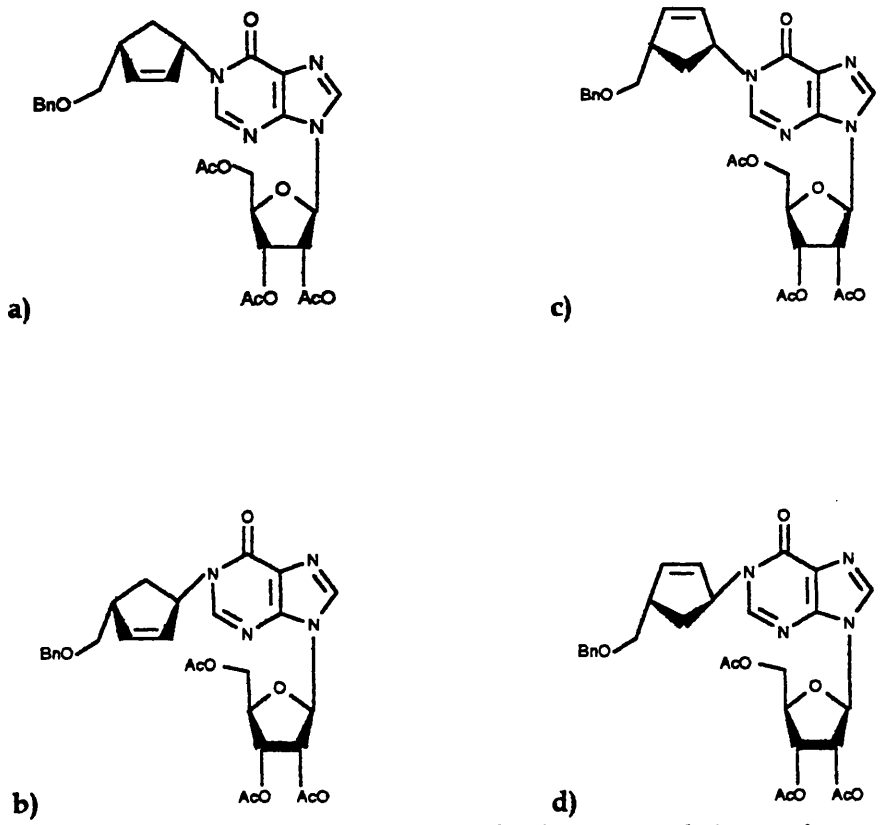


Figure 4.17 Stereoisomers of Bn-ether a-b) cis-isomers d-e) trans-isomers.
a) N^1-[(1S, 4R)-4-(benzyloxymethyl)cylopent-2en-1-yl]-2′,3′,5′-tri-O-acetylinosine.
b) N^1-[(1R, 4R)-4-(benzyloxymethyl)cylopent-2en-1-yl]-2′,3′,5′-tri-O-acetylinosine.
c) N^1-[(1R, 4S)-4-(benzyloxymethyl)cylopent-2en-1-yl]-2′,3′,5′-tri-O-acetylinosine.
d) N^1-[(1S, 4S)-4-(benzyloxymethyl)cylopent-2en-1-yl]-2′,3′,5′-tri-O-acetylinosine.

Modifier: Initial trials were conducted to find the most appropriate modifier for the separation of the Bn-ether on the (S)-NEC-β-CD column. The comparison of three modifiers is seen in Figure 4.18, in which the concentration of each modifier was chosen to produce approximately the same retention factor. For this, 30% MeCN, 20% 2-PrOH and 12% MeOH was required, however only 2 peaks were obtained when a sample containing all 4 stereoisomers was analysed. A sample containing the diastereoisomers a) and c) proved that the column separated the cis- and trans- isomer, however it was not capable of separating the enatiomer pairs a)/c) and b)/d). Figure 4.18 further shows that the separation with MeCN resulted in the best resolution, however the highest amount of modifier was necessary to increase the solvent power of the fluid, since MeCN is the weakest modifier.

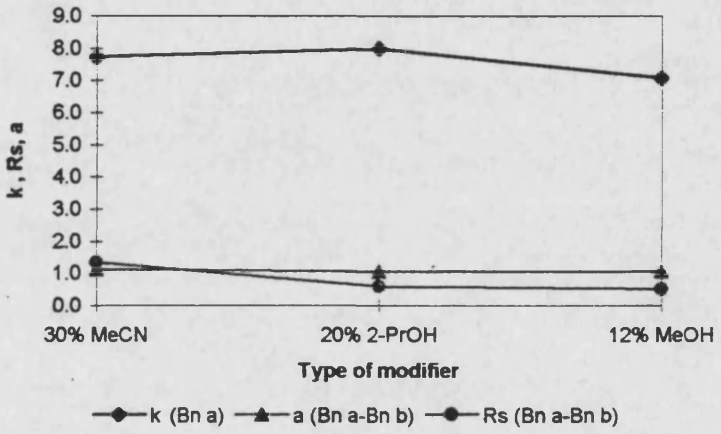


Figure 4.18 Retention factor, resolution and selectivity of Bn-ether using different modifers.
Conditions: (S)-NEC-β-CD, 55°C, 200kg/cm^2, 1.0ml/min.

In order to obtain the good resolution achieved with MeCN, while at the same time reduce the amount of modifier and retention time, trials were conducted in which small amounts of MeOH were added to MeCN. This was done with the expectation, that small additions of a stronger modifier would have a significant impact on the retention time, and at the same time would not reduce the resolution to a great extent. As indicated in Figure 4.19 the retention of the Bn-ether continuously decreased with the small addition of MeOH to 20% MeCN.

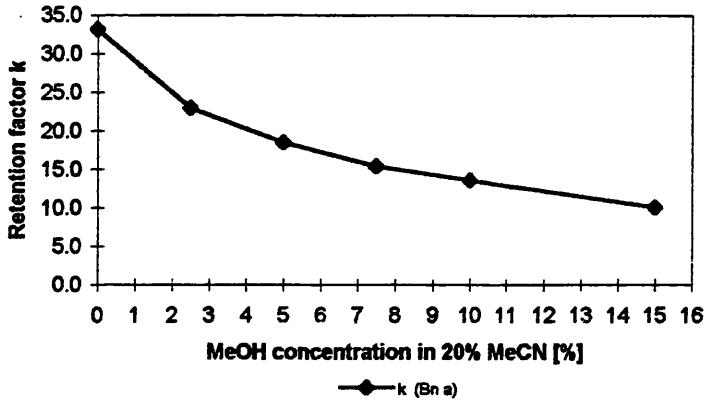


Figure 4.19 Influence of MeOH concentration added to MeCN modifer upon retention factor.
Conditions: (S)-NEC-β-CD, 55°C, 20% MeCN + MeOH, 200kg/cm^2, 1.0ml/min.

When the resolution of the compound was evaluated, a resolution enhancement with the addition of MeOH was observed and is shown in Figure 4.20. This result of increased resolution with marginally decreasing selectivity was surprising as it suggested that the efficiency of the separation increased, which was confirmed by the evaluation of the plate heights. The plate height for the 20% MeCN was 103μm and the plate height for 20% MeCN containing 10% MeOH was about 46μm.

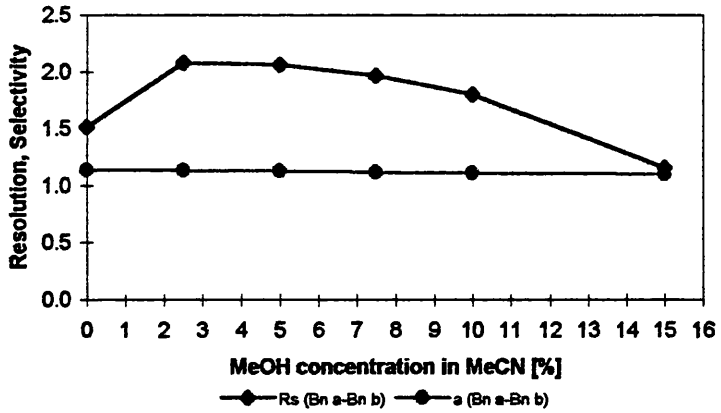


Figure 4.20 Influence of MeOH concentration added to MeCN modifier on resolution and selectivity.
Conditions: (S)-NEC-β-CD, 55°C, 20% MeCN + MeOH, 200kg/cm^2, 1.0ml/min.

An addition of 10% MeOH was chosen for further experiments, as this decreased the retention by 54%, however the resolution still increased by 16%.

Figure 4.21 demonstrates the separation achieved with the addition of 10% MeOH to the MeCN modifier. This also shows the baseline width broadening and it would be worthwhile to conduct the separation using 4.6mm i. d. columns to see whether the broadening was caused by extra column peak broadening.

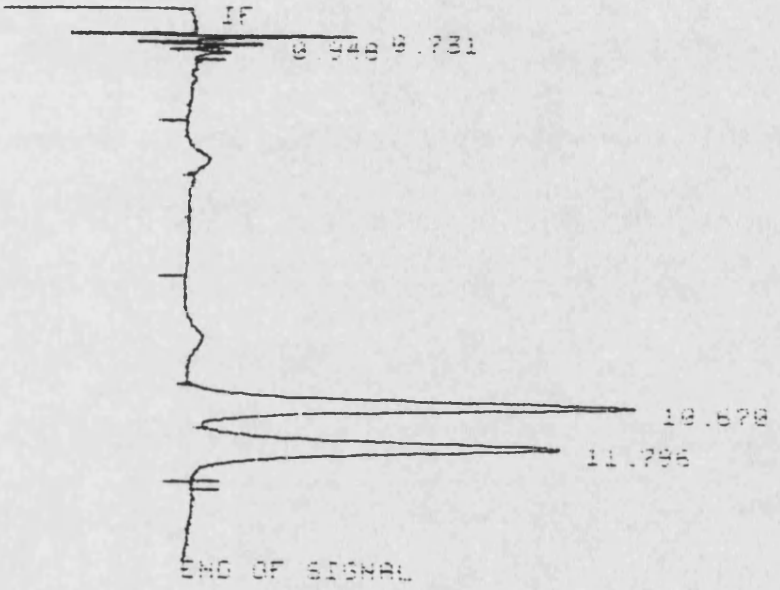


Figure 4.21 Separation of Bn-ether on a (S)-NEC-β-CD column.
Conditions: (S)-NEC-β-CD, 55°C, 20% MeCN + 10% MeOH, 200kg/cm^2, 1.0ml/min.

Since the separation was sufficient in terms of resolution, the MeCN was further increased to see whether it would be possible to achieve faster separation while still retaining sufficient resolution. However, an increase in modifier to 25% decreased the retention factor by 50%, and decreased concomitantly the resolution to 25%. Therefore, the optimum level of MeCN was considered to be 20%.

Temperature: Since temperature has normally the greatest influence on selectivity and resolution, the influence of temperature was investigated from 25-75°C. Figure 4.22 shows the retention factor, resolution and selectivity in dependence of temperature.

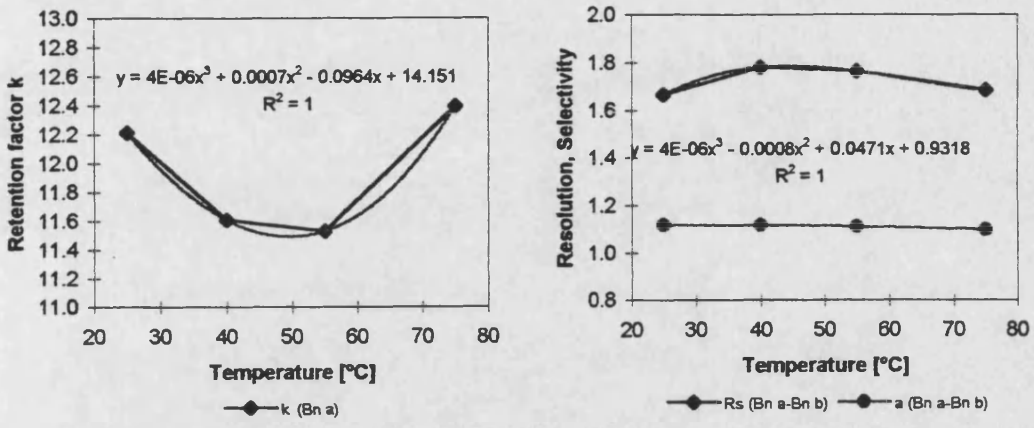


Figure 4.22 Influence of temperature upon the retention factor, resolution and selectivity of the Bn-ether separation.
Conditions: (S)-NEC-β-CD, 20% MeCN + 10% MeOH, 200kg/cm², 1.0ml/min.

Since the maximum value for resolution could not easily be identified from the Figure, the graphs were fitted to a polynomial curve and the resultant equation was used to calculate additional resolution values for different temperatures. The maximum resolution was obtained at 45°C, whereas the minimum retention factor was at 55°C. The investigation of pressure and optimum flow rate was therefore undertaken at 45°C.

During the temperature investigation, the separation at 55°C was different to that obtained while optimising the modifier concentration. The laboratory was colder when the temperature investigation was conducted, therefore more modifier was added relative to the mixture than in the modifier investigation, causing decreased retention and resolution, as listed in Table 4.7. In order to prove that the changed cylinder temperature was responsible for the changed separation,

the flow rate of CO_2 was increased from 0.8ml/min to 0.85ml/min, while the modifier flow rate was kept constant at 0.2ml/min. This resulted in the separation approaching that obtained while investigating the influence of the modifier concentration, therefore corroborating that the cause was the changing temperature in the laboratory. The temperature change could not be completely balanced by the cylinder thermostat, since the pump, which circulates water through the 60m pipe did not deliver a fast enough flow rate, hence the water temperature changed while flowing through the pipe.

Table 4.7 Variation of the separation due to changing temperature in the laboratory

Results obtained	k (Bn a)	Rs (Bn a - Bn b)
Modifier study	9.536	1.762
Temperature study	10.664	1.803
0.85 ml/min	10.396	1.798

Conditions: (S)-NEC-β-CD, 55°C, 20% MeCN + 10% MeOH, 200kg/cm^2, total flow rate 1.0ml/min.

Pressure: The pressure was varied from 150-250kg/cm^2 in order to investigate its influence on retention factor, resolution and selectivity. The retention factor changed by 36% over this range, the resolution by 9.5% and the selectivity by 0.5%. As before in the chiral separations the pressure appeared to be a far more efficient means to change the retention without excessive loss of resolution compared to changing the modifier. It is therefore recommended to use the highest possible pressure and then reduce the modifier to the required level to obtain a fast separation with sufficient resolution. The optimum pressure was chosen to be 175kg/cm^2 and used for the investigation of the flow rate.

Flow rate: As all the above optimisations were conducted at a flow rate of 1.0ml/min in order to accelerate the optimisation, the influence of flow rate on retention factor, resolution and selectivity in the range of 0.5-1.0ml/min was investigated. Figure 4.23 demonstrates clearly that the retention factor and the

resolution reduced significantly at flow rates higher than 0.6ml/min and therefore 0.6ml/min was the optimum flow rate for the separation.

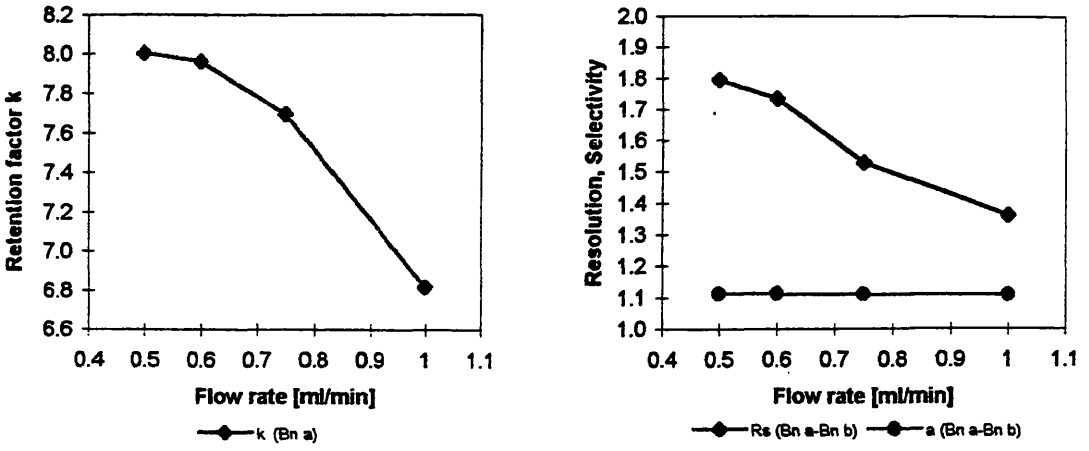


Figure 4.23 Influence of flow rate on the separation of Bn-ether
Conditions: 45°C, 20% MeCN + 10% MeOH, 175kg/cm^2, 1.0ml/min.

The Bn-ether was additionally separated on the silica, diol and β-CD column, however no separation of the cis/trans isomer was achieved on the silica and diol column. Separation was accomplished on the β-CD column, although not to the same extent as on the (S)-NEC-β-CD column. This can be seen in Figure 4.24.

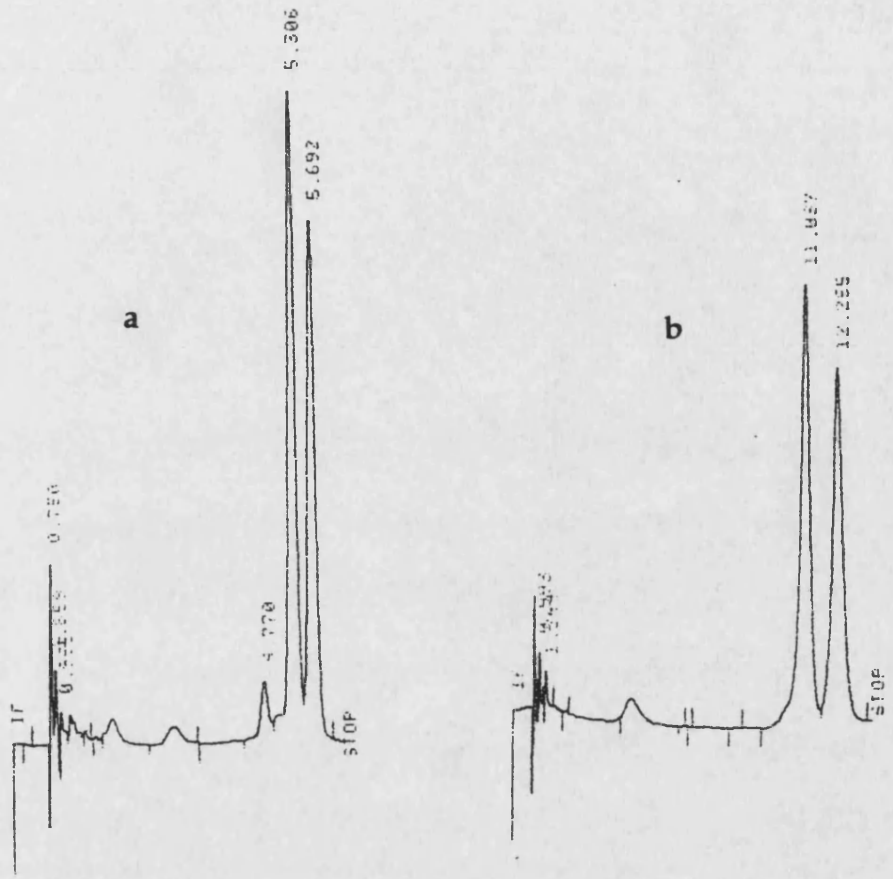


Figure 4.24 Separation of Bn-ether on a) β-CD and b) (S)-NEC-β-CD column
Conditions: 45°C, 20% MeCN + 10% MeOH, 175kg/cm^2, 1.0ml/min.

CHAPTER 5

SUPERCRITICAL FLUID EXTRACTION

5.1 Supercritical Fluid Extraction of Fatty Acids

Soya and cotton seed meals are frequently used as feeds for fermentation processes producing antibiotics, detergents and vitamins. The analysis of the feed is an important factor for quality control and hence consistent yield. Furthermore knowing the compositional makeup of the meals enables studies to be made on the effects which individual components may exert on the yield. Free fatty acids are one of the components which could influence the fermentation, and therefore a method for determining free fatty acid levels and composition could help to elucidate their importance on fermentation yields.

5.1.1 Introduction

The efficiency of SFE for the extraction of seeds has already been demonstrated by several researchers (Friedrich et al. 1982, Stahl et al. 1980, Taylor et al. 1993, King et al. 1992). Taylor et al. (1993) investigated the accuracy and precision of SFE for the determination of oil content in oilseeds, such as soyaflakes, canola flakes and corn germ. The same group of researchers (King et al. 1992) also developed a rapid on-line SFE-supercritical fluid reaction-capillary gas chromatography method for the analysis of fatty acid compositions of triglycerides and free fatty acids in oilseeds. However, this method did not independently measure the level of free fatty acids. This section describes the optimisation of a method to determine free fatty acids in soya and cottonseed meals.

The selection of optimum operating parameters is often a laborious task when one parameter is optimised at a time. Several theoretical approaches for predicting the solubilities of compounds have been published (King 1989, Mitra et al. 1991). These require physico-chemical data which are often not readily available. Another approach for the estimation of solubility is the use of chromatographic means (Bartle et al. 1990b, Smith et al. 1987). However, these theories do not include the effect of using modifier with the supercritical fluid hence the modifier effect must still be determined by experiment.

Several statistical experimental design approaches have been used for the optimisation of parameters involved in SFE. Mills et al. (1993) and Fisher (1989) used a simplex optimisation scheme to find the optimum of 2 extraction parameters. Bicking et al. (1993) used a full factorial design approach to optimise temperature and pressure and plotted the results in three dimensional plots which were beneficial in gaining an overall understanding of the influence of these parameters on the extraction. The optimisation scheme in this study used an statistical design software which allowed the optimisation of three factors and also provided three-dimensional plots, in which one of the parameters was kept constant.

The method reported here concentrates on free fatty acid profiles in cottonseed and soya meals, as the oil and hence the meal quality deteriorates when stored for a prolonged period prior to being processed (Marshall et al. 1991). This is easily detected by the increase in free fatty acids in the oil. As there is still about 5 % oil present in the meal, the decrease in meal quality can be monitored by determining the free fatty acid levels in the meal and comparing it with superior meals which have not deteriorated in storage and hence contain less free fatty acids.

5.1.2 Static versus Dynamic Extraction

Initially several extractions were performed on cottonseed meal 1 in order to compare recoveries using different modifiers, flow rates and the combination of static and dynamic extraction. Cottonseed meal 1 was chosen as it contained the highest level of free fatty acids. The extracts were derivatised as described in 2.3.3 and the sum of the fatty acids was used to estimate the overall recovery, namely: myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acids. The extraction conditions and recoveries are listed in Table 5.1.

Table 5.1 Conditions and recoveries used in the pretrials

Conditions	Flow rate [ml/min]	Modifier 5 mol %	Recovery [%]
A 30 min dynamic		methanol	77.6
B 5 min **15 min static** **10 min dynamic**		methanol	81.9
C 5 min dynamic **15 min static** **10 min dynamic**	3.0	2-propanol	75.7
D 10 min dynamic **10 min static** **10min dynamic**	3.0	methanol	81.2
E 10 min dynamic **10 min static** **10min dynamic**	2.0	methanol	77.6
F 10 min dynamic **10 min static** **10min dynamic**	4.0	methanol	77.6

50 °C, 200 kg/cm^2.

Methanol seemed to be a better modifier than 2-propanol, however in order to judge the significance of the difference between the two modifiers, replicate extractions (n=6) were performed to estimate the standard deviation. Conditions D in Table 5.1 were chosen for this experiment and the relative standard deviation was calculated to be ± 6.4%.

None of the conditions in Table 5.1 achieved any significant improvement compared to the rest as all the recoveries lay within the limits of the standard deviation. One can conclude from pre-trials that the extraction seems to be diffusion controlled rather than limited by solubility as no significant difference was determined when comparing the dynamic extraction mode with the static-dynamic combination.

If the extraction had been solubility limited then the amount of fluid used would have been important and hence the dynamic extraction would have shown a greater recovery. In the dynamic extraction 90ml of CO_2-methanol-fluid mixture were used, whereas in the static-dynamic combination only 40ml. Conditions E were then chosen for the optimisation experiments.

5.1.3 Optimisation with a Statistical Design System

The statistical design system was used to design a series of experiments enabling the optimum conditions to be found by performing only 15 experiments. The schematic design visualising the selected parameters is shown in Figure 5.1. This series of experiments includes one set of conditions with three replicates in order to determine the experimental uncertainty.

Cottonseed meal was used to conduct the experiments as spiked samples did not represent real samples. As shown in earlier experiments, the extraction seemed to be diffusion controlled and hence, the use of spiked samples would only give an optimum for solubility limited experiments and not for those limited by diffusion. Furthermore, spiked samples are not able to mimic active adsorption sites which influence extraction nor do they provide real distribution of the compound in the sample (Hawthorne 1983).

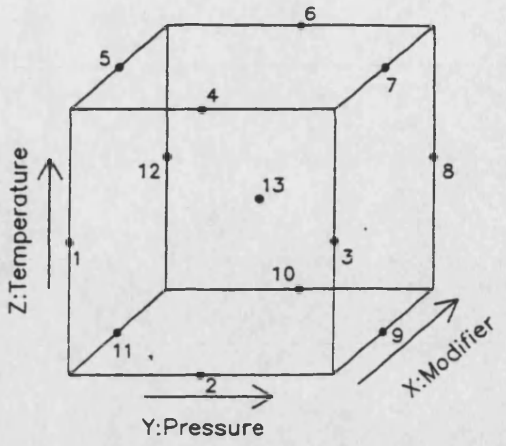


Figure 1 Schematic diagram showing the 13 experiments. Experiment 13 was carried out in triplicate.

The SFE unit was rebuilt to avoid long transfer lines outside the temperature controlled environment. The UV detector was not used to monitor the extraction as this may have caused degradation of the fatty acids, so the outlet from the extraction cell was connected directly to the backpressure regulator. The extraction mode used Condition E, Table 1 and the experimental conditions used for the extractions and their corresponding densities and solubility data are listed in Table 5.2.

The extraction temperatures ranged from 40-100°C, the pressures from 100-300kg/cm^2 and the modifier concentrations from 0-10mol%. The extraction recoveries for each of the 15 experiments are also listed in Table 5.2.

The results were used to fit a quadratic model and the following equation was used for calculating the polynomial:

$$= x_0 + x_1 A + x_2 B + x_3 C + x_{11} A\hat{}2 + x_{22} B\hat{}2 + x_{33} C\hat{}2$$

$$+ x_{12} AB + x_{13} AC + x_{23} BC \qquad \text{(eq. 5.1)}$$

the x values are the coefficients and the letters A-C represent the modifier, temperature and the pressure respectively. The values were calculated by the statistical design system and show the influence of each parameter on extraction yield.

Table 5.2 Extraction conditions and recoveries for the 15 experiments using the statistical design approach

Modifier [mol %] [a]	Temperature [°C]	Pressure [kg/cm²]	Density [g/ml] [b]	Solubility Parameter [b]	Recovery [%]
5	70	200	0.710	6.274	94.9
0	40	200	0.848	7.227	53.3
5	40	100	0.768	6.784	93.5
10	70	100	0.357	3.260	90.6
5	70	200	0.710	6.274	89.0
10	70	300	0.830	7.590	94.7
5	40	300	0.926	8.184	88.2
0	70	100	0.248	2.110	2.1
0	100	200	0.470	4.002	19.2
0	70	300	0.790	6.735	56.4
5	70	200	0.710	6.274	91.8
10	40	200	0.889	8.128	90.2
10	100	200	0.561	5.125	94.6
5	100	100	0.195	1.723	4.2
5	100	300	0.686	6.063	98.4

[a] Experiments listed in order of completion.

[b] Density and solubility was calculated using the ISCO solvent solver.

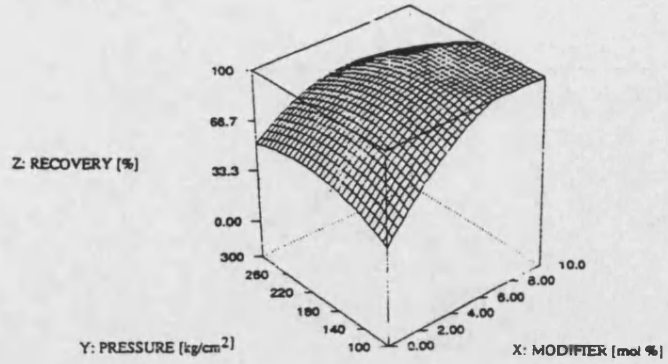
The calculated values were then used to present the data in three dimensional plots as seen in Figures 5.2(A)-5.4(A). Additionally, the solubility parameter δ_1 of the pure and mixed fluid was also processed using the statistical design system software to present the data in the same form as the extraction recovery, enabling comparison between the solvation power of the fluid and the resulting recoveries. The solubility parameter δ_1 was calculated by the "SF-solver" software using the equation derived by Giddings et al. (1968). This data was then fitted to a quadratic

model as this showed the best statistical correlation and is presented in Figures 5.2(B)- 5.4(B).

Figure 5.2(A) depicts the three-dimensional plot of the recoveries obtained with variations of pressure and modifier concentration at a constant temperature of 40°C. Looking at the front of the plot and following the increase of modifier at constant pressure, a steep ascent indicates a rapid increase in % recovery as the mol% modifier rises to 8 %. In the comparable solubility plot in Figure 5.2(B), this steep ascent is not present, and suggests that the solubility data is not representing the real change in solvent strength achieved by the addition of modifier. This discrepancy between solubility data and solvent strength has been noted previously (ISCO, 1991). Figure 5.2(B) depicts solvent strength in terms of density increase according to the solubility parameter theory, it therefore only shows the increase in solvent strength due to the increase in density caused by the addition of methanol and not the real increase in solvent strength. Therefore, Figure 5.2(A) shows the enhancing effect of methanol caused by the increased solvent strength, rather than Figure 5.2(B).

Deye et al. (1990) used the solvatochromic dye Nile Red to correlate mobile phase solvent strength to chromatographic retention in supercritical fluid chromatography. They found that a small addition of methanol to carbon dioxide produced a large increase in solvent strength causing non-linear plots of chromatographic retention versus % modifier. Better linearity was obtained when the retention data were plotted versus solvent strength determined with Nile Red. Rossi et al. (1990) further demonstrated the enhancing effect of ethanol on the extraction of egg lipids. The addition of 3% ethanol or more caused a considerable increase in extraction yield of cholesterol, cephalin and lecithin.

(A):



(B):

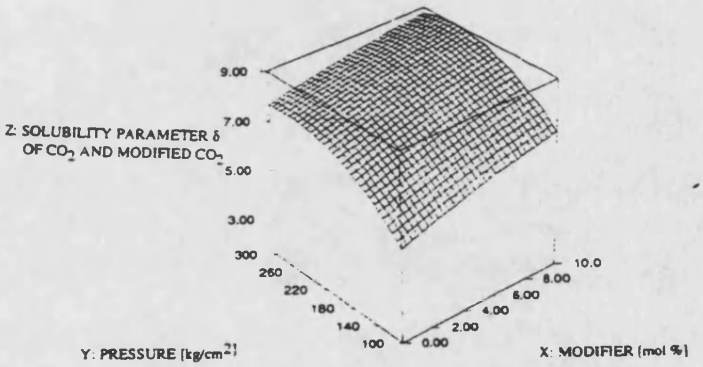


Figure 5.2 (A) Recovery of free fatty acids expressed in terms of the dependence upon modifier level and pressure at 40°C. (B) Variation in solubility parameter of CO_2 and methanol in dependence of modifier level and pressure at 40°C.

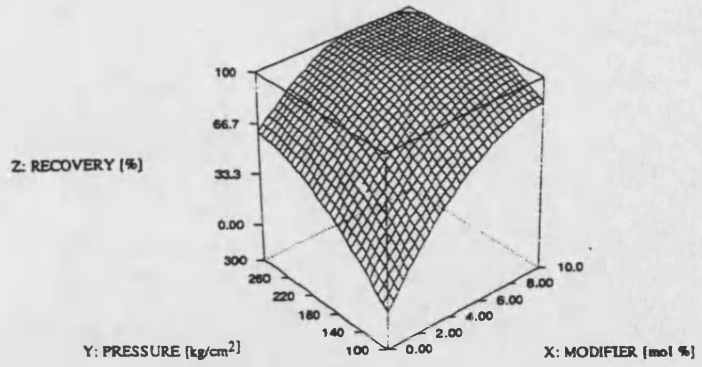
Figure 5.2(B) further suggests that the recovery should be at its highest when pressure and modifier are at high levels. Comparing this to Figure 5.2(A) however shows that the predicted recovery is actually lower in this region. This decline in recovery can be explained by assuming that once threshold pressure or maximum solubility is reached, extraction recovery is limited by diffusion of the analyte from the matrix to the bulk fluid. Therefore, a further increase in pressure beyond the threshold pressure or maximum solubility decreases the diffusion coefficient and

increases viscosity of the fluid resulting in the lower recovery. The decline in recovery is therefore caused by the change in viscosity and diffusivity and an increase of pressure beyond the pressure required for maximum solubility should therefore be avoided. The optimum conditions at 40°C appear to be with a pressure of 100-200kg/cm^2 and with a modifier concentration between 6-10%.

The temperature in Figure 5.3(A) was held constant at 70°C. Again the steep increase due to the addition of modifier is not represented in the solubility plot in Figure 5.3(B). Interestingly, the increase in recovery at 0% modifier with increasing pressure has almost the same ascent as in the solubility plot, proving that the solubility parameter is a valid estimate using pure carbon dioxide. The highest recoveries were achieved at high pressure and high modifier levels. The high level of these parameters are necessary to counteract the decrease in density compared to that at 40°C.

Figure 5.4 shows the three-dimensional plot of the recoveries at 100°C. Comparing Figures 5.2(A) - 5.4(A) at low pressure and low modifier levels, it is obvious that the 100°C plot shows the lowest recovery. The lower density and hence solvent strength of the fluid is responsible for this behaviour. Therefore, the temperature increase did not increase the vapour pressure of the free fatty acids to such an extent that it outweighed the decline in density.

(A):



(B):

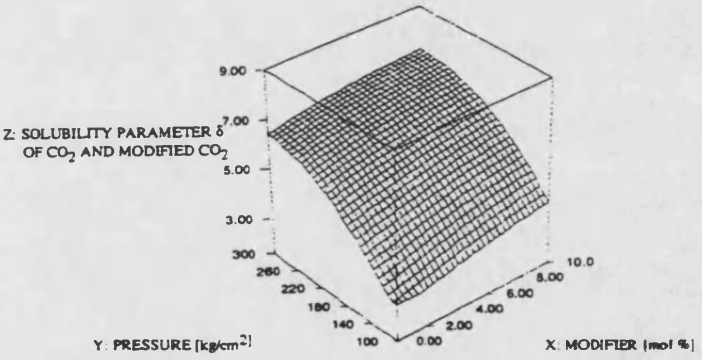
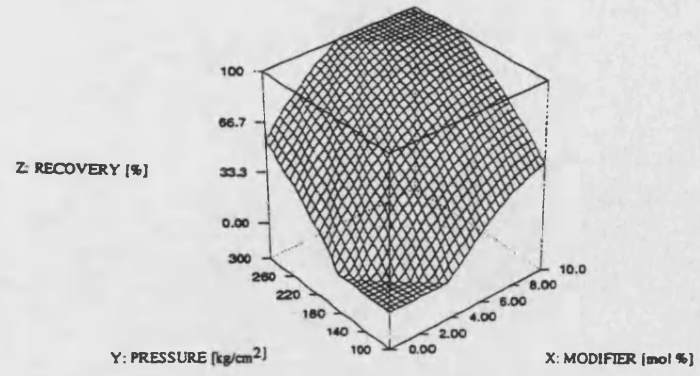


Figure 5.3 (A) Recovery of free fatty acids expressed in terms of the dependence upon modifier level and pressure at 70°C. (B) Variation in solubility parameter of CO_2 and methanol in dependence of modifier level and pressure at 70°C.

(A):



(B):

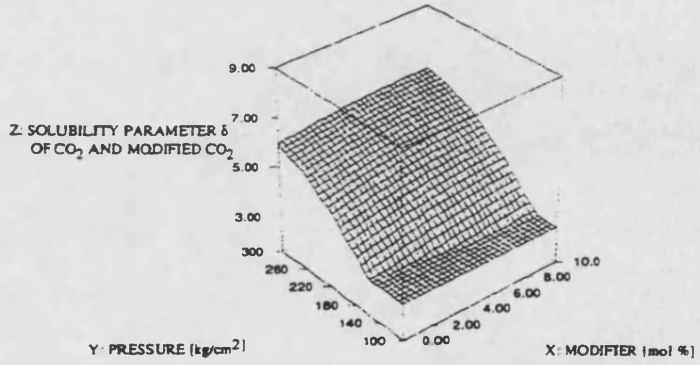


Figure 5.4 (A) Recovery of free fatty acids expressed in terms of the dependence upon modifier level and pressure at 100°C. (B) Variation in solubility parameter of CO_2 and methanol in dependence of modifier level and pressure at 100°C.

The three-dimensional plots also demonstrate the possibility of analyte fractionation as they depict areas of low and high recoveries. This knowledge can be used to extract compounds other than fatty acids using conditions at which none of the free fatty acids are extracted. The statistical design system allows the calculation of conditions at which fractionation is possible. Brunner et al. (1982) demonstrated fractionation of free fatty acids from triglycerides in which the

addition of ethanol had an enhancing effect on the fractionation. Fractionation was not of interest for the study reported here, as the triglycerides did not interfere with the analysis.

5.1.4 Validation of the Optimum Conditions.

The "expert" program offers the ability to estimate optimum conditions for extractions at different temperatures. Two experiments from each optimised region in the Figures 5.2(A), 5.3(A) and 5.4(A) were used to validate the optimum regions selected. The extraction conditions and recoveries are listed in Table 5.3 which shows that only one experiment (91.6%) had recovery outside the ± 6.4% standard deviation of 100.0% recovery. This outlier could have been caused by several reasons. First, low solubility of free fatty acids at these conditions. The density at which the low recovery was obtained is lower than the other experiments in Table 5.3. The density was obtained from experimental measurements published by Berger (1991). The "SF-solver" software however calculated a higher density at 100kg/cm^2 than at 150kg/cm^2. The "SF-solver" program did not calculate the density correctly because the temperature was below the critical temperature (T_c= 49.54°C) and hence subcritical conditions existed, at which the program fails to calculate the correct density.

Secondly, Strubinger et. al (1991B) measured the adsorption of methanol and carbon dioxide onto adsorptive material over a wide range of conditions. They found that a considerable amount of methanol is adsorbed in the near critical region and this additional layer of adsorbed fluid possesses a high density. Assuming that the analyte has to diffuse through this additional layer, in which its diffusion coefficient is lower than in the bulk fluid, could explain the lower recovery of the outlier.

Table 5.3 Conditions chosen for the validation of the optimum conditions[a]

Methanol [mol %]	Temperature [°C]	Pressure [kg/cm²]	Recovery [%]
8	40	150	97.3
9	40	100	91.6
7	70	280	96.1
9	70	250	99.3
6	100	290	97.0
8	100	280	99.6

[a] condition E in Table 5.1

Additionally it has to be considered that the conditions lay on the edge of the cube representing the experimental model (Figure 5.1) where statistical models often have the highest uncertainties. Therefore it is best to choose the optimum conditions from the middle of the calculated optimum region. Overall this proves that statistical experimental design is a valid technique of determining optimum conditions and provides useful information about the influence of each parameter.

5.1.5 Time-dependent Extractions

In order to determine the original amount (m_0) of free fatty acids in the meals, a time dependent extraction experiment using cottonseed meal 1 was carried out using the following conditions: 9mol% methanol, 70°C and 250kg/cm². The time intervals and the sum parameter of the fatty acid recovery are listed in Table 5.4. The results were used to calculate the original amount (m_0) of the fatty acids in the meal according to the hot-ball model which Bartle et. al (1990a) used as a model for dynamic extraction. This original amount was calculated to be 1.637mg/g and was used for the previous calculation of recovery. Table 5.4 lists the sum of extracted fatty acids at a certain time and the remaining amount of fatty acids using

1.637mg/g as the orignal amount. Figure 5.5 shows the corresponding graph in which $\ln(m/m_0)$ is plotted versus time.

Table 5.4 Sum of free fatty acids extracted during the time-dependent extraction and the calculated values of $\ln(m/m_0)$ for the plot in Figure 5.5

Time[a] [min]	Extracted free fatty acids FFA [mg/g]	Remaining amount m of FFA [mg/g]	$\ln(m/m_0)$
10	1.200	0.437	-1.321
15	1.507	0.130	-2.533
20	1.589	0.048	-3.529
30	1.606	0.031	-3.967
t_1 60	1.621	0.016	-4.628
t_2 120	1.629	0.008	-5.321
t_3 180	1.633	0.004	-6.014

[a] Condition E in Table 5.1.

The evaluation of the plot $\ln(m/m_0)$ gave a value of -3.49 for I and 66 minutes for t_c. The high value of I suggests, according to the theory, uneven distribution of the free fatty acids in the matrix or irregular particle shape. The theory further predicts that the linear portion of the curve should start at $0.5t_c$, which was calculated to be 33 minutes. As shown in Figure 5.5 the linear portion of the plot starts at around 30 minutes. Additionally, the recovery at $0.5t_c$ is normally around 50-70%, however about 98% was extracted at this time. The high recovery at $0.5t_c$ could be caused by the fact that the majority of the fatty acids are distributed in the outer part of the matrix and a higher percentage is extracted by the time a smooth concentration profile is achieved in the matrix. This demands maximum solubility and favourable diffusivity and viscosity at the beginning of the extraction to enable the fast mass transfer of the free fatty acids until equilibrium is reached at which the concentration is zero at the surface and reaches its maximum in the middle of the particle. From this point onwards the extraction begins to be limited by diffusion and an exponential decline can be observed in the plot $\ln(m/m_0)$ versus time.

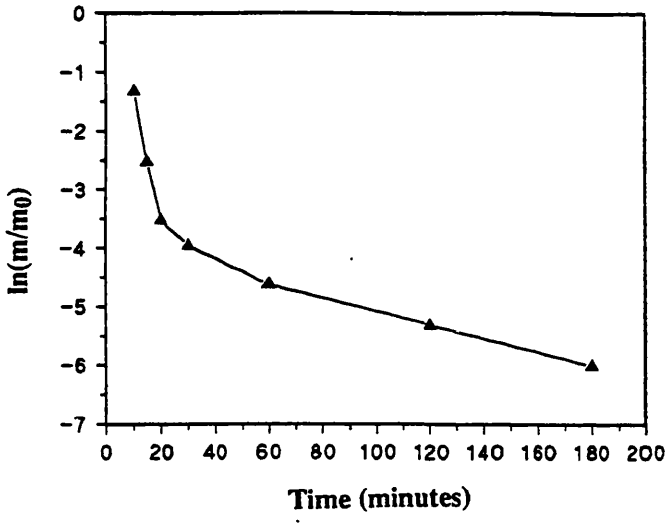


Figure 5.5 $\ln(m/m_0)$ vs time for the hot-ball model.

This also explains the results obtained from the initial comparison between dynamic extraction (Condition A, Table 5.1) and the dynamic-static combination (condition E). Once the majority of fatty acids were extracted the static extraction mode allowed the interparticle diffusion to take place and hence no flow through the cell was required. The subsequent dynamic step swept the fatty acids then to the collection vial. The time-dependent extraction could further determine the cause of the outlier in the validation experiment, since it distinguishes between extractions limited by diffusion or solubility.

5.1.6 Extraction of different Cotton Seed and Soya Meals

Finally, the optimum SFE conditions of 9mol% methanol, 70°C and 250kg/cm^2 were applied to extract two different samples of both cottonseed and soya meals using condition E (Table 5.1). The results were compared with liquid extraction using either petroleum ether or a chloroform : methanol-mixture as described in the experimental section. The results are listed in Table 5.5 and show that cottonseed meal 1 and 2 have considerably different levels of free fatty acids.

Table 5.5 Comparison of the sum of free fatty acid levels in different samples using different extraction methods

		Free fatty acid levels in mg/g					
Sample	Extraction mode	Myristic 14:0	Palmitic 16:0	Stearic 18:0	Oleic 18:1	Linoleic 18:2	Total
Cotton seed meal 1	SFE [a]	0.116	0.405	0.086	0.207	0.771	1.585
	Pet ether	0.088	0.262	0.074	0.161	0.553	1.333
	$CHCl_3$ [b]	0.109	0.336	0.081	0.189	0.683	1.400
Cotton seed meal 2	SFE	0.014	0.088	0.018	0.032	0.101	0.253
	Pet ether	0.010	0.039	0.020	0.019	0.063	0.151
	$CHCl_3$	0.014	0.051	0.016	0.022	0.087	0.190
Soya meal 1	SFE	0.009	0.292	0.025	0.133	0.102	0.562
	Pet ether	0.004	0.146	0.027	0.076	0.026	0.279
	$CHCl_3$	0.012	0.232	0.027	0.113	0.083	0.468
Soya meal 2	SFE	0.010	0.297	0.039	0.139	0.115	0.600
	Pet ether	0.005	0.116	0.022	0.065	0.024	0.232
	$CHCl_3$	0.012	0.271	0.027	0.138	0.107	0.556

[a] Condition E in Table 5.1. [b] Chloroform:methanol-mixture (2:1).

The pet ether extraction resulted in considerably lower recoveries, which is probably caused by the short extraction time of 2 minutes, which leaves no time for diffusion out of the matrix. The chloroform : methanol extractions achieved higher recoveries and are comparable to the SFE results. The longer extraction time and that the meal was extracted twice helped achieve higher recoveries. The results show the advantage of SFE for the extraction of fatty acids in cottonseed and soya meals, as SFE achieved higher recoveries and the filtration step needed for the liquid extraction was eliminated.

5.1.7 Summary of Fatty Acid Extraction

The statistical design system provided great assistance in gaining an understanding of the dependence of the different parameters in SFE. It showed the

increase in solubility due to the addition of methanol could not be predicted by the solubility parameter. The solubility parameter is however extremely useful when pure fluids are used assuming that no excessive adsorption of the analyte on the matrix takes place. In order to improve the usage of the statistical design system, it is advisable to determine rough boundary extraction conditions so that optimisation experiments need only to cover a small range of parameters and become more accurate.

Achieving maximum solubility was crucial for a fast extraction in this application as the analyte was present in a relatively high concentration compared to trace analysis, where solubility plays a minor role. Additionally, the fatty acids were mainly distributed in the outer layer, which required a high solvating power of the fluid in the beginning of the extraction. The comparison of SFE to liquid extraction showed that the SFE was faster, as no time-consuming filtration step was necessary, and higher recoveries were achieved. SFE for the determination of free fatty acids in cottonseed and soya meal minimises the use of solvents and additionally produces reliable results as seen from the low standard deviation.

The method represented here is applicable to other matrices, e. g. food, however a time dependent extraction should be conducted to estimate the required extraction time. This would then allow the calculation of the original amount of analyte in the matrix which can be compared with results obtained from conventional liquid extractions.

5.2 Extraction of Nicotine

5.2.1 Introduction

Since the Drug Abuse Advisory Committee has classified nicotine as an addictive substance (Kleiner 1994), this enables the US Food and Drug Administration to control and monitor the levels of nicotine in cigarettes and tobacco. There are several reports on the extraction of nicotine from tobacco using liquid extraction techniques (Saunders et al. 1981, Severson et al. 1981, Sudan et al. 1984), however since some of these suggest the use of aqueous buffers (Saunders et al. 1981, Sudan et al. 1984) subsequent liquid extractions are necessary to allow screening for pesticides using GC-MS.

The applicability of CO_2 for the extraction of nicotine from moist snuff has been demonstrated by Sharma et al. (1991) and the extraction of nicotine from tobacco as an industrial process has been patented (Roselius et al. 1979).

The extraction of nicotine from tobacco using the model of dynamic extraction has been used here to investigate the effects of particle size, extraction conditions, method of packing the cell, and cell geometry on extraction recovery profiles. These effects have been evaluated mathematically using the hot-ball model of Bartle et al. (1990a).

The recoveries using supercritical fluid extraction (SFE) has also been compared with "accelerated solvent extraction". "ASE" is a new technique developed by Dionex Ltd., in which solvents are used at elevated temperatures and pressures in order to increase analyte solubility and reduce solvent viscosity which improves solvent penetration into matrix pores.

5.2.2 Determination of initial Extraction Conditions

Initially several extractions, as described in section 2.4.5 Collection Efficiency were conducted to investigate the efficiency of the solvent trapping step. As pointed out by Hawthorne et al. (1993) it is crucial to test the trapping efficiency as low recoveries could be due to inefficient trapping. The experiments revealed that 3ml methanol trapped only 92.6% of the nicotine, therefore the amount was increased to 5 ml methanol which increased the trapping efficiency to 97.3%.

Tobacco (fraction 2b) and the following conditions were used to determine the most appropriate modifier: 8mol% modifier, 200kg/cm^2, 50°C, and 3ml/min total flow rate. Table 5.6 lists the recoveries which show that methanol and 2-propanol seem to be the most efficient modifiers. Additionally, the influence of water content on the extraction recoveries was investigated to see whether it is necessary to adjust the moisture level of the tobacco or whether the tobacco can be used as received. As seen from Table 5.6, for air-dry tobacco, methanol and methanol/water mixture were equally effective (11.08 and 11.22mg/g), and similarly for dry tobacco (13.62 and 13.39mg/g).

Table 5.6: Recoveries of nicotine in mg/g tobacco using different modifiers

Modifier	Tobacco fraction [µm]	Recovery [mg/g][a]
methanol	180 (air-dry)	11.08
2-propanol	180 (air-dry)	10.98
acetone	180 (air-dry)	9.12
acetonitrile	180 (air-dry)	10.14
methanol	180 (dry)	13.62
methanol/H_2O (97.5:2.5)	180 (air-dry)	11.22
methanol/H_2O (97.5:2.5)	180 (dry)	13.39

[a] recovery calculated on dry weight.
basis conditions: 200 kg/cm^2, 50°C, 3ml/min total flow, 8 mol% modifier, 10 minute extraction.

However, the higher recoveries of the dried tobacco after a 10 minute extraction are due to the drying process, which transports nicotine to the outer surface and makes it more readily extractable. This proves that the extraction conditions are sufficient and not solubility limited. If the extraction had been solubility limited, the same recoveries would have been obtained in both cases. Hence, for the subsequent time-dependent extractions the same extraction conditions, as above and methanol as a modifier were used.

5.2.3 Influence of Particle Size

For all the fractions listed in Table 5.7, time-dependent extractions were carried out to investigate the influence of particle size on extraction profile. The extracts were analysed and the results obtained were used to calculate $\ln(m/m_0)$. The initial amount was calculated by extrapolation using the model of Bartle et al. (1990a). Figure 5.6 shows the calculated value of $\ln(m/m_0)$ plotted versus extraction time.

Table 5.7 Levels of water and nicotine in different tobacco fractions

Tobacco Fraction [μm]	Fraction Number	Water [%]	Nicotine Level [mg/g][a]
125 - 180	1	10.0	18.02
180 - 250	2a	12.4	20.86
180 - 250	2b[b]	0	19.78
250 - 355	3	14.9	22.71

[a] determined by SFE, level calculated on dry weight basis
[b] fraction 2b was dried at 60°C for 24 hours

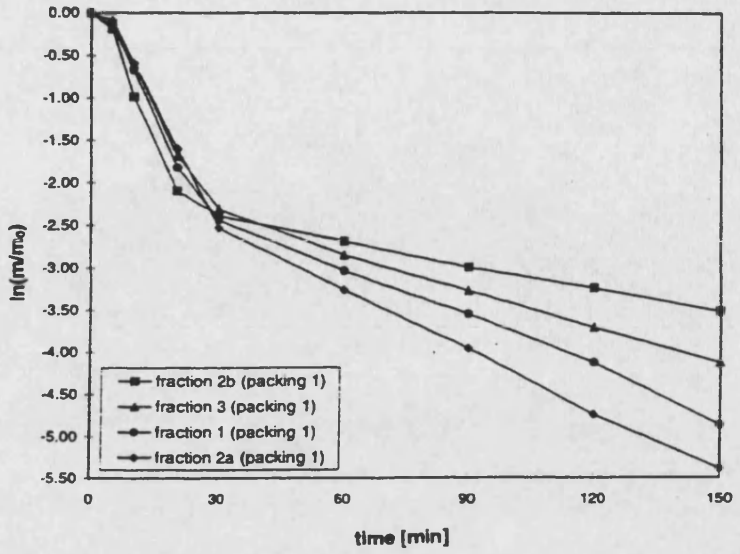


Figure 5.6 Dynamic extraction of nicotine by supercritical fluid CO_2 and methanol from tobacco prepared into four fractions as in Experimental (Table 5.7).
Conditions: packing 1 (Experimental), 200kg/cm^2, 50°C, 8mol% methanol.

Considering the results of the dried tobacco fraction 2b (180 - 250µm) first, the curve of $\ln(m/m_0)$ versus time has the form of the hot-ball model, characterised by its initial steep slope which becomes linear after a certain time. According to the model, the time at which the linear portion starts, should be $0.5t_c$. The slope of the linear portion is $1/t_c$ and can therefore be calculated. In the case of the dried tobacco it was calculated to be 52.6 minutes. However, the graphical evaluation gives a value of ca. 22 minutes. This indicates that the slope of dried tobacco should be steeper and hence diffusion out of the matrix seems to be a more complex process than assumed by the hot-ball model. This limitation has already been mentioned by Bartle et al. (1992a). The graph also shows by the initial steep fall that the dried tobacco yielded the highest recovery, which is caused by the drying process transporting more nicotine to the proximity of the surface. The high value of the intercept (I) confirms the non-uniform distribution of the tobacco, however this could also be caused by the particle shape being non-spherical.

Looking at the remaining graphs in Figure 5.6 one can evaluate the influence of the particle size on the extraction profile. Theoretically, it is expected that fraction 1 (125 - 180μm) should have the steepest slope and the linear part should be established in the least time. Using the mean radii of the different fractions the theory predicts a 2-fold increase in the slope when fraction 3 (r = 151.3μm) is compared to fraction 2 (r = 107.5μm) as listed in Table 5.8. The slope in the graph increases only 1.7 times, which shows quite good correlation to the theory. If fraction 1 (r = 76.3μm) is compared to fraction 2 one expects again a 2 fold increase for the smaller particle size, however one observed a 1.3 fold decrease. As already seen from the dried tobacco the slopes present a more complex process than diffusion through a homogenous matrix, therefore it seems the influence of the particle size is not the dominant factor in the extraction of nicotine. Moreover, after 30 minutes around 90% is extracted from all 4 fractions as seen from Table 5.8 and hence the sieving step can be omitted.

Table 5.8 Values characterising the linear part of plots of $\ln(m/m_0)$, obtained by calculation or graphical evaluation, for the different tobacco fractions

Tobacco fraction [μm]	Nicotine recovery [%][a]	Calculated slope [1/min]	Calculated $0.5t_c$ [min]	Visual value $0.5t_c$ [min]	Intercept (I) of linear part
125 - 180	91.2	0.0187	26.7	24.9	-1.886
180 - 250	92.0	0.0240	20.8	30.0	-1.815
180 - 250 (dry)	90.8	0.0095	52.6	22.3	-2.117
250 - 355	90.0	0.0142	35.2	32.6	-2.003

[a] after 30 minute extraction

Table 5.9 also lists the calculated times $0.5t_c$ at which the linear part should approximately start, however these do not correlate to the graphical evaluation. The graphical results appear to be more logical as they demonstrate that the largest particle took the longest time to establish the linear portion. Additionally, as the intercept (I) is influenced both by the non-uniform distribution and non-spherical particle shape no qualitative conclusions should be drawn.

Table 5.9 lists the recovery levels from fraction 2a during a time dependent extraction. Subsequently m_0 was calculated using the values in Table 5.8 and the following time intervals: m_1; t_1 = 0 - 30min., m_2; t_2 = 30 - 90min., m_3; t_3 = 90 - 150min., m_0 = 20.86 mg/g.

Table 5.9 Nicotine extracted from tobacco fraction 2a during the time-dependent extraction and the calculated values of $\ln(m/m_0)$

Time [min]	Extracted nicotine [mg/g]	Total amount of nicotine [mg/g]	Remaining amount of nicotine [mg/g]	$\ln(m/m_0)^a$
5	1.75	1.75	19.11	-0.088
10	7.55	9.30	11.56	-0.590
20	7.37	16.67	4.19	-1.605
30	2.52	19.19	1.67	-2.525
60	0.876	20.07	0.79	-3.269
90	0.397	20.46	0.40	-3.962
120	0.216	20.68	0.18	-4.747
150	0.086	20.77	0.09	-5.392

[a] m_0 = 20.86mg/g

The graph in Figure 5.7 confirms that m_2 and m_3 are taken from the exponential part. However, in order to show how the graph can be influenced by the value calculated for m_0, different time intervals were used to calculate m_0: m_1; t_1 = 0 - 60min., m_2; t_2 = 60 - 90min., m_3; t_3 = 90 - 120min., m_0 = 20.94mg/g. The graphs in Figure 5.7 demonstrates how a difference between the m_0 values (0.38%) influences the slopes. The slope for the m_0 value of 20.84mg/g is 1.2 times greater than the m_0 value of 20.94mg/g. Therefore, when interpreting the effects of different parameters on the slopes one has to take this uncertainty into account.

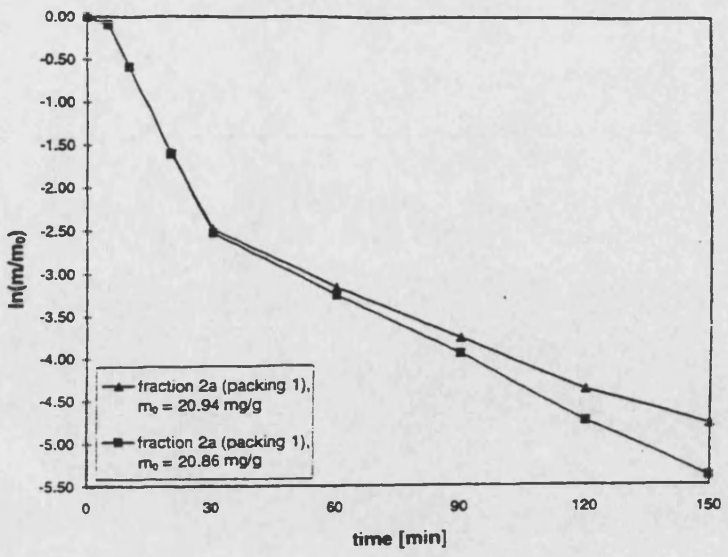


Figure 5.7 Influence of m_0 on the slope of $\ln(m/m_0)$ versus time.

5.2.4 Influence of Extraction Conditions

Fraction 3 (250 - 355μm) was used to investigate the influence of pressure and modifier levels upon the extraction efficiency and the calculated $\ln(m/m_0)$ are listed in Table 5.10. Increasing the level of methanol from 8 to 12mol% at 200kg/cm^2 did not increase the initial steep fall and it actually decreased slightly. This was caused however by the inhomogeneous nature of the sample rather than being influenced by the modifier level. Next, the modifier was kept at 12mol% methanol and the pressure increased to 300kg/cm^2. As seen in Table 5.10 the initial steep fall remained the same, indicating that maximum solubility was reached using 8mol% methanol, 50°C and 200kg/cm^2.

Table 5.10 Values characterising the linear part of plots of $\ln(m/m_0)$, obtained by calculation, for the different extraction conditions

Extraction conditions[a]	Nicotine recovery [%][b]	Calculated slope [1/min]	Intercept (I) of linear part
200kg/cm² 8mol% MeOH	90.0	0.0142	-2.003
200kg/cm² 12mol% MeOH	90.2	0.0187	-1.881
300 kg/cm² 6mol% MeOH	89.8	0.0159	-2.022

[a] 50 °C, 3 ml/min total flow rate.

[b] after 30 minute extraction.

An untreated commercial tobacco (0.5g) with a water content of 17.68% was extracted using the same conditions and the cell was packed according to packing 1. The graph $\ln(m/m_0)$ versus time is plotted in Figure 5.8, showing also the graph obtained from the extraction of tobacco fraction 3 for comparison. Theoretically, one would expect a less negative intercept (I) for the untreated tobacco as the nicotine should be more evenly distributed compared to the powdered sample.

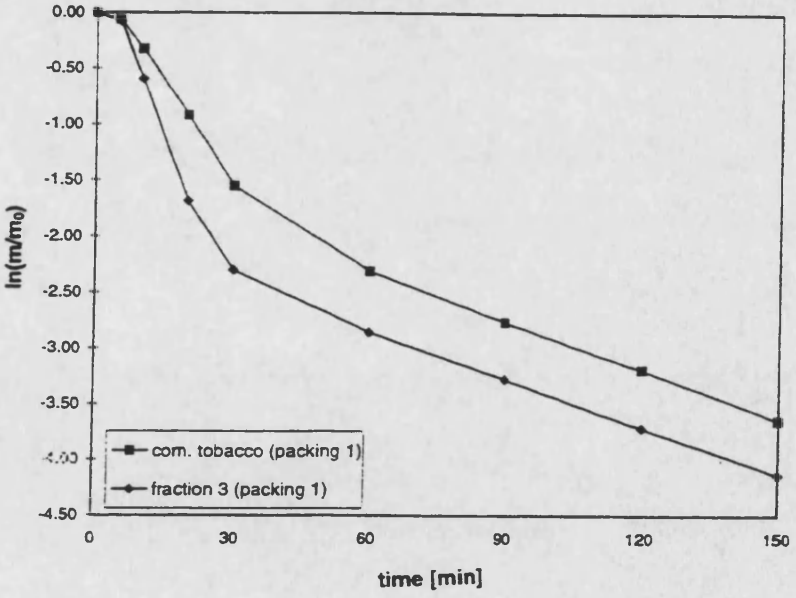


Figure 5.8 SFE of untreated and powdered tobacco (fraction 3).

At the same time, the untreated tobacco deviates more from the ideal sphere and hence this could cause a more negative intercept. However, this is only true if the surface-to-volume ratio increases (Bartle et al. 1990) which is not the case for the untreated tobacco as the strands used have actually a lower surface-to-volume ratio. Additionally, $0.5t_c$ should be larger as the linear part takes longer for larger particles to be established and the slope is expected to be flatter.

As seen from Figure 5.8 the intercept is less negative, hence confirming the more even distribution of nicotine in the tobacco matrix compared to the powdered tobacco. Therefore, the time needed to reach the linear part is greater compared to fraction 3 which is also in agreement with the theory. This means that if tobacco is extracted without prior treatment, the optimum extraction time is longer compared to the time necessary to extract the powdered tobacco. The optimum extraction time is the time at which the linear portion of the graph is reached, as the majority of the tobacco is extracted at that time and the extraction rate slows down considerably, which produces low standard deviations in replicate experiments.

5.2.5 Influence of Packing the Cell and Cell Geometry

In order to investigate the influence of the method of packing the cell, time-dependent extractions using packing method 1 and 2 (section 2.4.3) were carried out. Tobacco fraction 3 and the same conditions as earlier were used to conduct duplicate time-dependent extractions. As seen in Figure 5.9 packing 2 moved the graph downwards without changing the slope significantly.

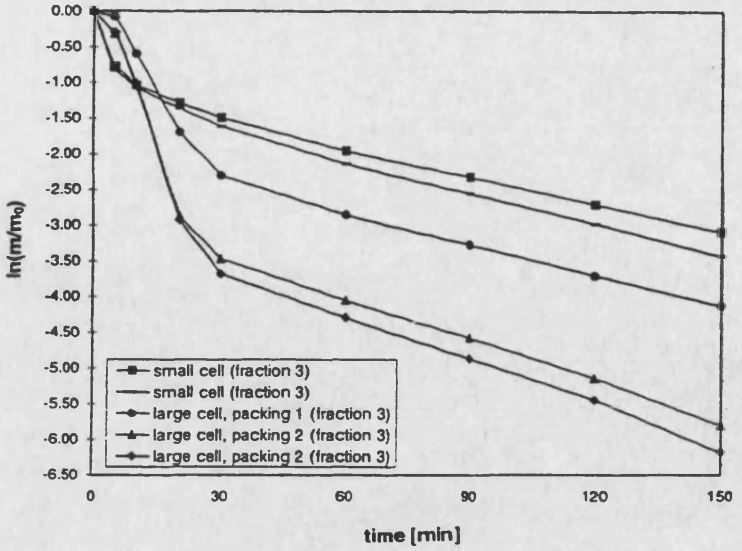


Figure 5.9 Influence of cell geometry and packing upon the extraction profile of nicotine from tobacco fraction 3.

The reason for this was that more nicotine had been extracted by the time the linear portion was reached, suggesting that the extraction using packing 1 was solubility limited. This however can be excluded as in both extractions the same extraction conditions were used. It seems as if the profile of the initial slope is influenced by the length of the distance the nicotine has to be transported before being flushed out of the cell. Hawthorne et al. (1993) reported insufficient flushing out of the extraction cell when the cell was horizontally connected or when vertically positioned having a large void volume and being extracted from bottom to top. All these factors can be dismissed as not occurring in this case, as the extraction cell was vertically mounted, the void volume was filled with α-cellulose and the extraction occurred from top to bottom.

However, α-cellulose with its hydroxyl groups interact strongly with nicotine via hydrogen bonding, causing the retention of the nicotine. Therefore, the additional α-cellulose in packing 1 after the tobacco is probably responsible for the slow desorption kinetics of the nicotine, which influences the amount being extracted

after 30 minutes. However, it leaves the time at which the linear part of $\ln(m/m_0)$ starts unchanged.

A small cell with an internal volume of 1.67ml was therefore used to perform replicate time-dependent extractions as before. The results are also plotted in Figure 5.9 and surprisingly the graph moved upwards. The shape of the initial steep slope changed and the same shape was predicted by Pawliszyn (1994) in his theoretic kinetic model when the flow rate was infinite, suggesting that the maximum amount of nicotine was recovered. The cell dimension of the 1.67ml cell is 10 * 0.46 cm, therefore the distance required by the nicotine to reach the cell exit is longer than with the 10ml cell (6.1cm * 1.45cm). The first interval recovered roughly twice as much nicotine compared to the 10ml cell as the linear velocity using the small cell is ca. 3 times faster. Therefore, the cell is flushed more often, causing the greater recovery in the beginning. However, as soon as the water present in the tobacco is extracted, the extraction rate slows down considerably. This means that after 30 minutes only 76.7% nicotine is extracted using the 1.67ml extraction cell compared to 90% with the 10ml extraction cell (packing 2).

5.2.6 Influence of Flow Rate

As the flow rate of 3ml/min using the 1.67ml cell did not accelerate the extraction, a lower flow rate using the 10ml cell was investigated to determine whether the flow rate can be reduced to 2ml/min. Time-dependent extraction were performed using tobacco fraction 3 with a flow rate of 2ml/min. Figure 5.10 shows the plot $\ln(m/m_0)$ versus time including the extraction with 3ml/min flow rate for comparison. The slopes of the two extractions using 2ml/min flow rate deviate quite considerable from each other which is caused by the inhomogeneous nature of the tobacco. It is clearly demonstrated that the initial steep fall is less pronounced when the flow rate is reduced and additionally that $0.5t_c$ is greater and shows the theoretical features of a solubility limited extraction, which are a

reduction at the beginning of the extraction, a decrease in the slope and to move the slope upward on the graph (Cotton et al. 1993).

The flow rate of 2ml/min was not sufficient to achieve to reach the linear part in the least time. Therefore, higher initial recoveries could be achieved with the 10 ml cell using flow rates larger than 3ml/min. However, the use of 4ml/min flow rate causes the trapping efficiency to decrease. An experiment using 4ml/min flow rate to test the trapping efficiency revealed that only 91.4% was trapped. The increase in flow rate would further only be beneficial for the first interval as in the subsequent intervals the flow rate does not influence the recovery. A flow rate of 3ml/min seemed therefore the optimum flow rate for 0.5g tobacco.

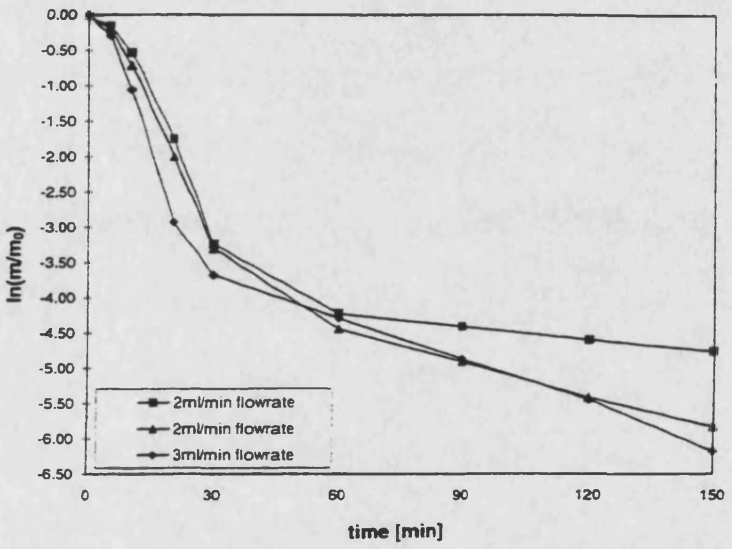


Figure 5.10 Influence of flow rate on the extraction profile.
(fraction 3, packing 2).

5.2.7 Influence of Water Content

The initial experiments (Table 5.6) led to the conclusion that there was no difference between the extraction of air-dry and dried tobacco when using the

large cell packed with α-cellulose. The water content of the α-cellulose was determined by drying at 100 °C to constant weight and showed that the α-cellulose had a water content of 2.76%.

Therefore 0.069g water from the α-cellulose were present in the extraction of dried tobacco in Figure 5.6 and this amount seemed to be sufficient to yield the same recovery as the extraction of air-dry tobacco, which has a total water content of 0.130g, within 10 minutes. This leads to the assumption that the lower recovery in the beginning using packing 1 compared to packing 2 (Figure 5.9) must be caused by the presence of additional α-cellulose, which introduces more adsorption sites. Addtionally, when tobacco fraction 3 was extracted using pre-extracted α-cellulose, the recovery of nicotine after 30 minutes was about the same as using the small cell. One can therefore conclude that water is necessary to allow the shortest possible extraction time.

In order to achieve the same recovery with pre-extracted α-cellulose, the water content had to be increased to 5v/v% in the methanol when 8mol% modifier was used. Using this mixture for the extraction of tobacco held in the small cell one would expect the same extraction profile for the small cell as with the large cell (packing 2). Figure 5.11 shows the extraction profile of tobacco fraction 3 when 5vol% water is added to the modifier using the small cell and and for comparison the extractions using the large cell and the small cell without water addition to the modifier. The extraction profile using the small cell changed dramatically and 97.5% is recovered after 30 minutes which is the same as with the large cell.

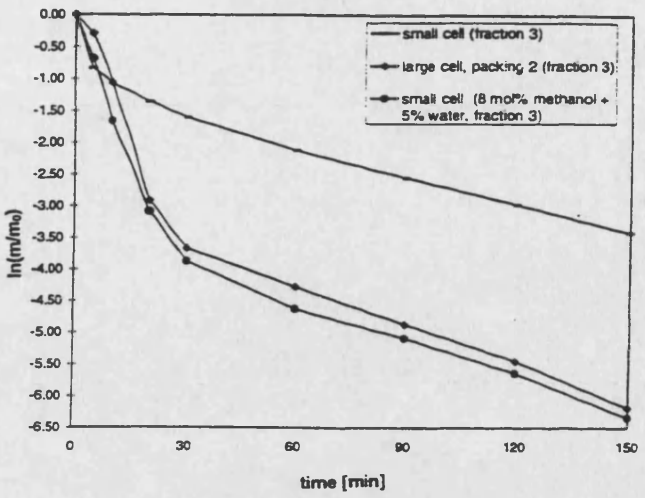


Figure 5.11 SFE of nicotine from tobacco fraction 3 using the large cell (packing 2) and the small cell.

5.2.8 Constituents of Tobacco

As water and the addition of α-cellulose have a great influence on the recovery, this can be used to selectively extract constituents of tobacco such as flavourings, pesticides and other contaminants. Additionally, around 50% less nicotine is recovered when dried tobacco is extracted with methanol as modifier without further water addition.

Screening for pesticides in tobacco is necessary and will be even more important when transgenic tobacco plants are introduced, which are resistant to the herbicide bromoxynil and higher levels can be applied to the crop without damaging the plant (MacKenzie, 1994). In order to screen for a wide range of constituents, tobacco fraction 3 was extracted using the large cell (packing 2). The collected extract was evaporated and dissolved in 200 µl of dichloroethane and analysed by GC-MS. The following compounds were identified using the reference library: 2-methylphenol, 3-methylphenol, 2,4-dimethylphenol, 3, 4-dimethylpenol, 4-ethyl-2-methoxyphenol, dehydroacetic acid (*), 2,6-bis(1,1-dimethylethyl)-4-methylphenol

(*), 2-ethyl-1,1`-biphenyl, [R-[R*,R*-(E)]]-4-(3-hydroxy-1-butenyl)-3,5,5-trimethyl-2-cyclohexen-1-one, [S-(E,Z,E,E)]-3,7,11-trimethyl-14-(1-methylethyl)-1,3,6,10-cyclo-tetradecatetraene. Two of the above substances (*) are listed as pesticides in the reference library. Benzoic acid could be identified in the extract when ethylacetate was used as solvent.

5.2.8 Comparison of different Extraction Methods

As seen from the graphs, the linear part using both the small and large cell (packing 2) is established after 30 minutes and hence using 30 minutes dynamic extraction recovers the majority of the nicotine. Good standard deviation should be achieved with repetitive extractions. Table 5.11 lists the recoveries after 30 minutes obtained in the time-dependent extractions using the extrapolated values of m_0 as reference values.

Table 5.11 Recoveries of nicotine after 30 minutes extraction

Tobacco	Packing	Recovery [%]
1	1	91.2
2a	1	92.0
3	1	90.0
3	2	97.5
3	small cell	77.4
3	small cell	80.0

Subsequently, 6 extractions each using the small and large cell were performed with the following conditions: 8mol% methanol, 50°C, 200kg/cm^2 and a flow rate of 3.0ml/min. Additionally, 6 liquid extractions according to Saunders et al. (1981) and 3 extractions using the new "accelerated solvent extraction" method using fraction 3 and the solvent listed in 2.4.8 were conducted for comparison. 'ASE' is new extraction technique developed by Dionex and involves the use of high

temperature organic solvent extraction at raised pressure. A basic introduction into 'ASE' was given by Richter et al. (1996).

As seen from Table 5.12 the SFE using both cells and liquid extraction showed excellent correlation. However, "ASE" showed higher relative standard deviation which may be due to the short period for temperature equilibration and that the purging with CO_2 was not as efficient as high pressure purging with nitrogen as carried out by Dionex.

Table 5.12 Recoveries of nicotine using different extraction methods

Method	No. of Repetitions	Recovery [mg/g]	Rel. Standard Dev. [%]
SFE[a]	6	22.71	1.38
SFE[b]	6	23.06	2.08
Liquid extraction	6	22.63	2.14
ASE	3	21.35	2.93

[a] 10 ml extraction cell (packing 2).
[b] small cell.

5.2.10 Summary of Nicotine extraction

Use of the hot-ball mathematical model allowed the calculation of the original mass, m_0, of nicotine in the tobacco sample, hence allowed optimum extraction times to be determined. The comparison of these results with liquid solvent extraction showed excellent correlation.

The resulting graphs however have to be evaluated carefully in order not to draw wrong conclusions, as the model does not account for all parameters influencing the extraction. Bartle et al. (1992a) pointed out that real SFE systems are complex in terms of geometry, solute distribution, and the effect of the matrix on the extractability of the solute. A comprehensive treatment would therefore be daunting (Bartle et al. 1992a). Nevertheless, the model is an excellent help in the calculation of the initial mass. If more than one compound (e.g. pesticides) needs to

be determined the hot-ball model allows after an initial time-dependent extraction, the initial amounts of each compound to be calculated.

The experiments showed that the particle size had no major influence upon 30 minutes extractions as after 30 minutes 92.0% nicotine was extracted from fraction 2 and 90.2% nicotine from fraction 3 and 91.1% from fraction 1. One can conclude that no time is gained by sieving the tobacco, whereas grinding achieved a 10% higher recovery and hence is beneficial. Time-dependent extractions must be carried out initially in order to calculate the original mass of nicotine in a particular type of tobacco.

The experiments also showed that it is important to determine the water content of samples and any additives (eg. α-cellulose) added to the extraction cell. The influence of these parameters can be used to introduce additional selectivity and hence makes SFE very attractive for selective determination of compounds.

5.3 Practical Problems during Use of SFC/SFE System

5.3.1 Control of Cylinder Head Pressure

One of the major problems encountered was the changing temperature of the laboratory, influencing the headpressure of the CO_2 cylinder and hence the delivered flow rate. When mixed mobile phases were used, this caused a change in concentration relative to each other, therefore creating mobile phases with differing composition. The variation in capacity factor caused by the changing mobile phase composition was clearly seen in section 3.2 while analysing the alkaloid analogues. The installation of a pressure regulating valve was not possible, as no company could be traced which manufactured valves operating at pressures of about 60 - 70kg/cm^2. The problem was then alleviated by regulating the

temperature of the CO_2 cylinder, however as the flow rate of the pump circulating water through the 60m braced PVC tubing was not efficient enough, the temperature still deviated when temperature differences were severe.

5.3.2 Control of the Pump Head Cooling

When extractions were conducted, the extraction cell was pressurised using flow rates of 5ml/min so that the pressurisation step did not take up a significant proportion of the timed extraction step. A temperature probe was inserted into the cooling block and an increase in temperature was measured during the pressurisation step where a fast flow rate was used. The problem of cooling the pump head was twofold. Firstly, the cooling bath pump works on flow resistance, which meant that the colder the coolant became, the slower the flow through the cooling jacket due to the increasing resistance in flow with increasing viscosity. Secondly, even though the tubing for the coolant was configured to be as short as possible, the distance to the pump was still considered too long. The tubing was therefore thoroughly insulated to avoid unnecessary heating up of the coolant. The cooling jacket used for this work had the disadvantage of not enclosing the checkvalves, which would be recommended for improved valve activation. However the checkvalves and the top and the underside of the pump were covered with insulation material to avoid loss of cooling efficiency. The cooling bath was then set to -10°C, which enabled the temperature on the pump head to be between -5 to -3°C.

5.3.3 Life Expectancy of Pump Seals

Initially, the pump seals lasted at the most only 6 weeks and then required changing, as they started to leak when the pressure check in accordance with the pump manual and described in section 2.25 was performed. Rinsing the seals with MeOH often extended their life time for a further 2-3 weeks. The failure of the

seals was associated with the build up of black material on the pistons, which was initially thought to stem from contamination of the CO_2, however since the inlet filter did not show any build up this appeared to be unlikely. Additionally, when the pump head was removed, release of trapped CO_2 could be observed, which was finally traced back to the expansion of piston guides inside the pump head. These guides were made of rule-on material and appear to have contained extractable material which deposited on the pistons causing damage to the seals. Following replacement of these guides with PEEK guides, no further change of pump seals were required.

5.3.4 Mixing efficiency at low Flow Rates

The initial set-up of the SFC system contained only the large HPLC column (25cm * 8.0 mm i. d.) which was filled with a Teflon rod to operate as a flow dampener and mixer. However, using the 2.1mm i. d. columns, flow rates at 1ml/min and below were used, which caused the baseline to become noisy when more than 5mol% MeOH was used. The installation of a Jasco mixer Pu 880-30 resulted in a smooth baseline, however when gradients were run at flow rates of below 1ml/min the baseline disturbances were again visible. Not even the combined mixing of the Jasco static mixer and a dynamic mixer from Gilson (1.5ml volume) were able to produce a smooth gradient profile. This indicated that improvement in mixing efficiency at low flow rates was required. It would be desirable to achieve the mixing in low dead volume device as otherwise the gradients would take a long time to reach the column. Furthermore, the modifier pump which delivered very small volumes (0.05ml/min) at these low flow rates may not be capable of delivering small volumes efficiently due to the design of the check valves and pump set up.

Ashraf-Khorassani and Levy (1995) used a microbore reciprocating pump to deliver small amounts of modifier and Saito and Takeuchi (1989) developed a

cascade pump for accurate flow delivery when using compressible fluids. It would therefore be valuable to determine if the noisy baseline was caused by the pump or the mixing step.

The integrated pulse dampener inside the pumps consists of a large cylinder filled with a Teflon rod. It has been observed that CO_2 was released even after all the cylinders and valves had been closed. Closer investigation revealed that CO_2 was absorbed onto the Teflon inside the pulse dampener, which may suggest that extraction of compounds from the Teflon rod could be possible. This may contaminate collected fractions and cause problems in subsequent analysis or makes an on-line connection to a GC-ECD impossible.

5.3.5 Oven Set-up

The set-up of the oven could be improved as the injection valve was mounted on the oven door. The opening and closing of the door resulted in continuous stress on the valve connections of the injection valve. In addition the connecting tubing from the valve to the column experienced continuous stress from the movement of the door, requiring the connections in this tubing to be replaced far more frequently than other non-movable connections. This problem could be easily avoided by installing the injection valve on the top of the oven.

A further limitation of the oven was its limited maximum temperature of 100°C, as Langenfeld et al. (1993) demonstrated the enhanced extraction efficiency at 200°C. However, when working at the maximum of 100°C, it was necessary to wear insulated gloves, as during installation of the extraction cell the contact with the oven could not be avoided. In general, the installation of cells and columns could be more user friendly and the oven should be equipped with a cool down system as incorporated in GC ovens.

CHAPTER 6

CONCLUSIONS

6.1 Alkaloid Separation

In order to analyse the extent to which each of the physical parameters influenced the retention factor, resolution and selectivity, changes in these chromatographic parameters were evaluated for each column. The results are collated in Table 6.1 and the calculations applied were described in section 2.2.7.

Temperature: Solubility in a fluid is governed by the chemical and state factors as defined by Giddings (1969), extraction and distillation by Zosel (1978) and by the free volume and chemical interaction by McHugh and Krukonis (1994). All three definitions describe the same two effects, expressed in different terms. Increasing the temperature increases the volatility of the analyte, thus enhancing one part of the solubility as the phases of fluid and analyte become more alike, which is expressed as state effect, free volume or distillation effect. Simultaneously, the other factor relating to solubility decreases due to the fact that chemical interaction between the fluid and the analyte are reduced at higher temperatures. This is described as the chemical effect or extraction effect. An increase in retention can be observed if the chemical effect dominates the state effect. However, retention on a column is not only governed by the solubility of the analyte in the mobile phase but also by the interaction in the stationary phase.

Table 6.1 Overall comparison of differences in chromatographic parameters when fluid conditions are changed

Parameter	Range	Physical Condition	k (Nic)	k (Cot)	k (Ana)	k (Nor)
				Change in k		
(S)-NEC-CD						
Temperature [°C]	40-100	15% Modifier, 200kg/cm²	43	63	-38 (28)	33
	40-70				-49 (33)	
	40-80				-56 (36)	
	40-90					31
Pressure [kg/cm²]	300-150	60°C, 10% modifier	52	60	49	57
Modifier [%]	10-5	60°C, 200kg/cm²	34	67	63	
Modifier [%]	15-10	60°C, 200kg/cm²	11	32	29	45
Flow rate [ml/min]	0.3-1.0	60°C, 10% modifier, 200kg/cm²	6	6	-1 (1)	3
β-CD						
Temperature [°C]	40-100	10% Modifier, 200kg/cm²	49	68)	-24 (20)	47
	40-70				-43 (30)	
	40-80				-48 (33)	
	40-90					43
Pressure [kg/cm²]	300-150	60°C, 10% modifier	61	64	52	56
Modifier [%] 40°C	15-5	200kg/cm²	-28 (22)	46		61
Modifier [%] 40°C	15-10	200kg/cm²	-4 (4)	21	32	41
Modifier [%] 60°C	15-5	200kg/cm²	-1 (1)	56	52	
Modifier [%] 60°C	15-10	200kg/cm²	-3 (3)	27	26	45
Modifier [%] 100°C	15-5	200kg/cm²	42	70	58	
Modifier [%] 100°C	15-10	200kg/cm²	11	36	25	43
Flow rate [ml/min] 40°C	0.3-1.0	10% Modifier, 200kg/cm²	11	12	8	11
Flow rate [ml/min] 60°C	0.3-1.0	10% Modifier, 200kg/cm²	15	15	7	15
Diol column						
Temperature [°C]	40-100	8% Modifier, 200kg/cm²	65	77	-7(6)	29
	40-70				-46 (32)	
	40-80					
	40-90					34
Pressure [kg/cm²]	300-150	50°C, 8% modifier	53	59	49	53
Modifier [%]	15-4	50°C, 8% modifier, 200kg/cm²	15	63	61	70
Modifier [%]	15-10	50°C, 8% modifier, 200kg/cm²	7	28	29	41
Flow rate [ml/min]	0.3-1.0	50°C, 8% modifier, 200kg/cm²	15	11	11	10

Berger (1995) pointed out that using the argument of volatility is not logical, as only one phase exists. The decrease in retention with increasing temperature was

explained in terms of desorption of CO_2 and MeOH from the stationary phase. This decreases the volume and polarity of the stationary phase, which then results in a decrease in k. However, this does not explain why certain compounds increase in retention with increasing temperature.

Since the hydroxyl groups on the diol and β-CD column are considered to have the same ability for dipolar interaction (Technicol 1992), the same degree of retention change with increasing temperature would be expected for the diol and β-CD column if the same interactions were dominant. However, as the mobile phase used for the diol column contained a lower percentage of modifier this may have influenced the retention behaviour to a greater extent. Indeed, changes in retention factor for nicotine and cotinine with increasing temperature was greater for the diol column and the least on the (S)-NEC-CD, where the highest concentration of modifier was present in the mobile phase. The assumption that the modifier was the dominant factor, to which extent the retention factor varied with temperature was supported by the results on the β-CD column, which can be seen in Figure 6.1. It is clearly demonstrated that at lower modifier concentrations the retention of cotinine was influenced to a greater extent by temperature than at higher modifier concentration.

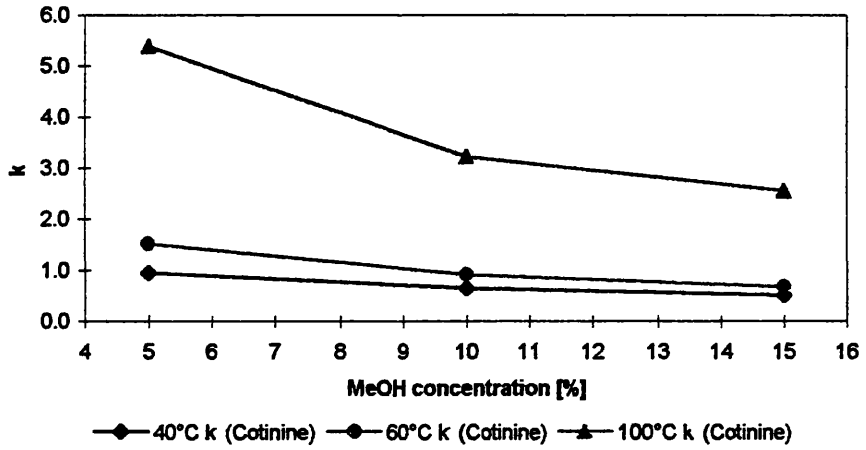


Figure 6.1 Influence of modifier concentration on the retention behaviour at different temperatures.

The Figure 6.1 further explains the disproportionately greater increase on the diol than on the β-CD column, since the retention appeared to increase more with a modifier change of 10-8% on the diol column than with a modifier change from 15-10% on the β-CD column. The retention factor of anabasine decreased to a far greater extent on the (S)-NEC-CD column which was must be due to the higher solvent strength of the mobile phase, which enhanced the solubility with increasing temperature.

Therefore in order to be able to distinguish between the effect of solvent strength and decrease in interactions with temperature on different columns, the trials need to be repeated under the same conditions in order to make a quantitative judgement. The conditions in this experiment were chosen so that the retention factors were about the same to allow better comparison of efficiency.

The lowest increase of nornicotine retention (40-90°C) was expected to be on the (S)-NEC-CD column, due to the higher solvent strength of the mobile phase. However, the changes on the diol column were the least, which was surprising and contradictory to the previous observations. The marked increase of the nornicotine retention with temperature could be easily rationalised as the solubility decrease with temperature, however this was not observed to the same extent on the diol column, despite the lower modifier concentration. A possible explanation might be the desorption of some of the amine additive, exposing residual silanols and thus causing increased retention of nornicotine. However, it was reported by Smith et al. (1995) that hydrogen bonding interactions decreased substantially with temperature, making the above assumption less possible.

Pressure: The changes with pressure produced similar changes in retention factors for anabasine and nornicotine on all three columns, but the greatest changes for nicotine and cotinine were observed on the β-CD column. This

suggested that the interactions between the analytes and the stationary phase were changed to the same degree on all three columns independently of the modifier concentration. The only exception was nicotine on the β-CD column, as its retention was significantly different on the other two columns.

Modifier: Nornicotine eluted with a very bad peak shape and irreproducible retention times at 5% modifier, therefore the modifier influence was only investigated from 10-15%. The same changes in relative retention were observed for cotinine, anabasine and nornicotine on all three columns. The same was observed with a change in pressure, therefore it was concluded that a change in modifier concentration did not influence the interactions between analytes and stationary phase, but merely caused a preferential partitioning into the mobile phase. The retention of nicotine on the β-CD column deviated from the rest.

Flow rate: The influence of flow rate on the retention factors of nicotine and cotinine was comparable on all columns, however the retention factors of anabasine and nornicotine were more influenced by the flow rate on the diol and β-CD column.

In order to discuss which parameter was the most efficient in bringing about a change in retention factor, resolution and selectivity on each column, the parameters changing the chromatographic parameters to the greatest extent are listed in Table 6.2. In order to assess the modifier influence, the range of 5-15% was used in all cases, except for the retention factor of nornicotine, where 10-15% was used. For the evaluation of modifier on the β-CD column the modifier concentration at 60°C was used, in order to have a comparison to (S)-NEC-CD and diol column.

Table 6.2 Importance of physical parameter in changing retention factor, resolution and selectivity

Column	k (Nic)	k (Cot)	k (Ana)	k (Nor)	Rs (Nic-Cot)	Rs (Cot-Ana)	Rs (Ana-Nor)	α (Nic-Cot)	α (Cot-An a)	α (Ana-Nor)
Parameter changing retention factor, resolution and selectivity to the greatest ectent										
(S)-NEC-CD	Pressure	Modifier	Modifier	Pressure	Modifier	Tem-perature	Tem-perature	Modifier	Tem-perature	Modifier
β-CD	Pressure	Tem-perature	Modifier	Pressure	Tem-perature	Tem-perature	Tem-perature	Modifier	Tem-perature	Modifier
Diol	Tem-perature	Tem-perature	Modifier	Modifier	Modifier	Tem-perature	Tem-perature	Modifier	Modifier	Tem-perature

As can be seen from the Table, although the flow rate had the least influence on retention factor and selectivity, it nevertheless had a major influence on the speed of analysis and efficiency. Berger (1995) noted that in order to change retention, the percentage of modifier was the most important, followed by pressure, temperature and flow rate. However, as demonstrated in Table 6.2, the importance of any physical parameter in changing retention depended on the compound. However, it must be noted that the modifier range was only considered within a very narrow range. Modifier may have shown to be the most efficient parameter in changing retention, if a wider range of modifier concentrations had been investigated. Berger (1995) noted that the most important parameters in influencing selectivity was primarily temperature, followed by pressure and modifier. Contrarily, for the separation of alkaloids, modifier and temperature appeared to be more important. The difference between Berger (1995) and this study was due to the different nature of the analytes and the retention mechanism. The guidelines suggested by Berger (1995) appear to be appropriate for a majority of compounds, however as demonstrated here there are exceptions. For developing methods in SFC it is therefore important to assess the changes induced by varying physical parameters and then decide on the most efficient.

6.1.2 Future Work

Further trials investigating the adsorption of amine on the stationary phase are necessary and it would be interesting to determine the amount of adsorbed amine and modifier on different stationary phases. Additionally, it should be determined if the amine is completely eluted from the stationary phase within a reasonable time scale, since it was indicated that it took over 60 minutes to elute the amine completely when 20% MeOH was used in the mobile phase. It was also noted that 8% MeOH was unable to elute the amine. Testing the presence of amines after a determined rinsing time with a binary fluid mixture of CO_2 and MeOH could be done by injection of acidic and neutral compounds in the SFC mode or by rinsing the column with an appropriate solvent on a HPLC system and subjecting the rinsing solvent to NPD analysis. The injection of acidic and neutral compounds would be however the more convenient test. This test could additionally be used to confirm the complete elution of one amine, for example butylamine before the retention behaviour and efficiency of other amines such as triethylamine or diethylamine are investigated.

It is not only important to investigate whether separations are feasible using SFC, it is even more important to investigate the reproducibility of the developed method. If the retention of a certain compound class is very susceptible to changes in running parameters, then these have to be controlled most carefully, or another analysis technique has to be chosen. As seen from the discussion of irreproducible results, there is a great need to control the temperature of the CO_2 cylinder or to control the outlet pressure of the CO_2 cylinder, which would be more efficient. The retention times and therefore the selectivity of anabasine and nornicotine were more variable than that of nicotine and cotinine and it required about an hour to achieve constant selectivity of anabasine and nornicotine indicating that stronger amines as additives were necessary for these compounds. It is therefore not practical to separate compounds such as anabasine and nornicotine using SFC, if no additive is available which is more basic than these two compounds to ensure a

controlled retention mechanism. The limitation of SFC is therefore conditioned by the strength of available additives and their removal from the column.

Since it was impossible to elute all four alkaloids from any of the investigated columns using only MeOH as modifier it was not feasible to interface the column effluent to a nitrogen-phosphorus detector in order to achieve lower detection limits for the four alkaloids. However, in toxicological studies, in the main, the less polar analogues such as nicotine and cotinine are of interest. These were shown to elute from the diol column, therefore it would be worthwhile to investigate their detection limits on a NPD detector using a MeOH modified mobile phase. The addition of amine would increase the baseline noise, therefore anabasine and nornicotine could not be analysed with such a set-up. Before this can be undertaken, the set-up of the SFC system has to be improved to provide a system which does not suffer from all the factors discussed in 3.1.

To sum up, the separation of the alkaloids on the various phases clearly demonstrated the flexibility of SFC and the ease of influencing the chromatographic parameters. However, the susceptibility of retention factor, resolution and selectivity on the physical parameter was at the same time the biggest drawback of SFC, since the parameters have to be controlled precisely to obtain reproducible results.

It was further evident that in order to obtain fast equilibration in SFC the mobile phase additives have to be stronger bases or acids in order to control the retention mechanism. Additionally, it was necessary to confirm if all amine was rinsed off the column. If the column is irreversibly changed by the use of amines, then the column should only be used for a specific method or the applicability of SFC for a certain application has to be reassessed.

It is therefore not recommended to develop methods using SFC, if other methods are far more applicable for certain separations, as SFC may show an uncontrolled retention mechanism. SFC has proven its advantages in many

applications, it is therefore not necessary to find unrealistic applications for the sake of justifying SFC in the analytical laboratory, since this only brings SFC into disrepute.

6.2 Separation of PCBs

6.2.1 Separation According to Planarity

Separation of PCBs according to planarity was demonstrated using the (S)-NEC-β-CD column, however separation did not appear to be solely based on planarity, but also on the electron distribution of the aromatic ring and steric factors. To assess the real potential of the separation power of (S)-NEC-β-CD column more individual PCBs need to be studied, as the fractionation of Arochlor 1260 highlighted that a number of PCBs eluted with the mono- and non-ortho substituted PCBs. PCB80 and 81 which are known to be less toxic for mammals, showed reduced retention on the (S)-NEC-β-CD column, which indicated that toxicity might be directly related to the retention behaviour. McKinney (1984, 1985) confirmed there is a structure-activity relationship for PCBs. The idea of toxicity was then hoped to be applicable for a range of compounds, but Lee et al. (1996) proved that toxicity cannot be mimicked by interaction strength with the Ah receptor. Nevertheless, the ratios of retention factors were calculated for (S)-NEC-β-CD and β-CD, diol and silica column in order to ascertain whether this corresponded to the toxicity of the PCBs. None of the ratios was able to correspond with the measured toxicity for all the investigated PCBs, however a group division into ortho-, mono- and non-ortho substituted PCBs was possible for the individual PCBs investigated. The fractionation of a Aroclor mixture however revealed that a number of PCBs were present in the mono- and non-ortho substituted group despite being non-planar and assumed not to be toxic. Therefore the suitability of

the column for fractionation and toxicity evaluation of PCBs needs to be confirmed by investigating an extended range of individual PCBs.

Possibly a more attractive method of separating planar from non-planar PCBs in a single analysis would be the production of a (S)-NEC-β-CD GC column so as to allow a direct resolution of the PCBs according to their planarity by means of a GC column. Hess et al. (1995) reported about the lack of a GC column capable of separating the PCBs according to their planarity, which would make a fractionation into separate classes using different techniques obsolete. Cyclodextrin phases have been used in GC applications (Technicol 1992). Although the (S)-NEC derivative has not yet been produced for the use in GC, probably due to thermal stability and derivatisation problems. It would be interesting to investigate this possibility.

6.2.2 Separation of PCBs from Fat

In order to judge which approach is the most suited for the separation of PCBs from fat, there should be dialogue with laboratories, where real samples are analysed on a routine bases, to develop a method which is most suited for daily use. The following options could be considered for the separation of PCBs from fat: on-line transfer of separated PCBs from the fat either a) on-line to a GC equipped for example with HT8 column from SGE or similar high temperature non-polar column, which is able to separate most of the 209 PCB congeners, b) on-line transfer to a SFC system, which is able to separate the PCBs according to their planarity and then either off-line collection or on-line transfer to a GC or c) off-line collection of the PCBs and then off-line analysis either with prior separation according to planarity or direct GC analysis or d) separation of fat on-line in the SFE extraction step, if SFE is used for the extraction of PCBs.

An off-line system has the advantage that the processing of the resultant sample could proceed as soon as the remaining fat was eluted from the column. In an on-

line system however, the slowest step would be rate-determining, which means that the processing of a new sample could not proceed until the previous sample had been completely analysed.

The separation of Witepsol S55, which is commonly used as a suppository base, indicated the possibility of analysing either the quality of the fat or it could be used for the analysis of suppositories containing drugs. If the chiral columns (S)-NEC-β-CD and β-CD columns are used, chiral separation could be conducted at the same time if the suppository contained enantiomers. However, contacting laboratories in which these analysis are performed on a routine bases would give insight as to what is needed in terms of analysis of suppositories.

In this thesis it was further demonstrated that PAHs can be separated from oil or fat within 6 minutes and therefore offers a quick method for separation. There needs to be further information about the type of oil in which the determination of PAHs would be necessary. Additionally, since the oil needs to be eluted with MeOH or other modifiers before the next analysis, the equilibration times of the column needs to be established in order to confirm the applicability of this method within a routine laboratory.

6.3 Comparison of Chiral Results

6.3.1 Results obtained on β-CD

In order to judge the effects of the physical parameters upon the chromatographic parameters the changes in the chromatographic parameters were calculated and are listed in Table 6.3.

Compounds P5 and P6 are amines, whereas C7, C8 and C11 possess a carboxylic group. It was therefore surprising that the increase in amine concentration caused a decrease in retention factor for all the compounds, as an increase in retention of the clofibrate analogues was expected due to increasing ionisation. However, ion-pairing must have occurred and therefore this increased the solubility in the tertiary fluid mixture.

The amine concentration changed the retention factor of C7 and C8 by 30% for an amine concentration range of 0.5-1.0% and for C11 by 14.0% for a range from 0.75-1.0%. This means that if a linear behaviour is assumed, the retention factors would change by about 45% for an amine range of 0.25-1.0%. Comparing this with the results of the propanolol analogues, where the amine concentration had only a comparatively minor effect, the assumption can be made that an ion-pair mechanism is responsible for the retention of the clofibrate analogues.

A change in modifier concentration was the most efficient way to control retention, which corresponds with the findings of Berger (1995), however this influenced the resolution and therefore the efficiency of the separation to a considerable extent. The change in modifier concentration however had only a minor effect on the selectivity.

Table 6.3 Overall comparison of the influence of different physical parameters on retention, resolution and selectivity

Chromatog. Parameter	Modifier conc.	Amine conc.	Temperature at const. pressure	Temperature at const. density	Pressure
	7.5-20%[c]	0.25-1.0%[a]	22-30°C	22-30°C	100-250kg/cm²[b]
	Change in chromatogr. Parameters [%]				
k (P5a)[d]	-49.2	-13.6	+3.4	-26.4	-31.6
k (P6a)	-48.3	-12.1	+3.9	-25.9	-32.5
k (C7a)	-33.6	-28.6	+1.3	-28.5	-26.7
k (C8a)	-36.2	-32.4	+4.2	-23.8	-24.8
k (C11a)	–	-14.0[f]	+2.3	-25.1	–
R_S (P5a- P5b)	-27.2	-2.0	-22.6	-35.4	-3.0
R_S (P6a- P6b)	-33.0	-4.2	-19.0	-31.7	-0.2
R_S (C7a- C7b)	-16.8	-3.9	-11.4	-19.3	-8.5
R_S (C8a- C8b)	-16.9	-8.5	-11.4	-17.4	-9.0
R_S (C11a- C11b)	–	+29.2[f]	28.2	-39.2	–
α (P5a- P5b)	-0.3	-0.3	-1.4	-1.1	-0.4
α (P6a- P6b)	-0.4	-0.3	-1.6	-1.4	-0.6
α (C7a- C7b)	-0.5	0.0	-1.0	-1.1	-0.2
α (C8a- C8b)	-0.3	-0.3	-1.0	-1.3	-0.4
α (C11a- C11b)	--	+0.9[f]	-1.0	-1.5	--

[a] for C7, C8 from 0.5-1.0% iso-PA.
[b] for P5, P6 100-200kg/cm² and for C7, C8, C11 150-250kg/cm².
[c] for P5, P6 7.5-15% and for C7, C8, C11 15-20%.
[d] refers to the first eluting enantiomer.
[f] for C11 0.75-1.0% iso-PA.

The amine concentration had only a minor effect on the resolution of the propanolol analogues, although it had a significant effect on the clofibrate analogue C11. In contrast to the resolution of the other compounds the resolution of C11 improves with increasing amine concentration. This indicated that the amine might

have suppressed undesired interactions of the solute (C11) and the stationary phase. However, since the amine also influenced the enantioselectivity, it could be that it had an effect on the retention mechanism. Additional studies with different amines, such as that used for the alkaloid investigation should be used in order to elucidate the function of the amine in this chiral recognition process.

Changing temperature at constant pressure was less well defined since the retention decreased from 22-25°C and then increased from 25-30°C due to the loss in solvent power of the fluid with decreasing density. At the same time the strength of the interactions between the CSP and analytes decreased, causing a decrease in resolution and selectivity. The same, although more predominately, could be observed for the temperature investigation at constant density. The retention decreased due to the sole effect of temperature in reducing the interaction between the CSP and the analyte, since the density was kept constant. Berger (1995) explained this with decreasing absorption of mobile phase components on the stationary phase and therefore decreasing volume and polarity of the stationary phase, causing the decrease in retention.

The pressure increase caused a significant reduction in retention factor over the investigated range, however little effect on the selectivity was noticed. Moreover, the pressure increase hardly affected resolution, which means that retention can be reduced without compromising the efficiency of the separation.

To sum up, temperature at constant density was the parameter of choice for influencing selectivity. In order to modify the retention, a change in pressure appeared to be the most efficient method, since it did not deteriorate resolution to the same extent as a change in modifier concentration.

6.3.2 Results obtained on (S)-NEC-β-CD

Mourier et al. (1987) proposed the presence of an inclusion process in SFC, however the results were compared to NP-LC separations, in which no inclusion complex takes place. The retention mechanism for the (S)-NEC-β-CD column appeared to be very similar to the β-CD column in terms of elution order, however retention was enhanced on the (S)-NEC-β-CD column to a greater extent, which must have been due to π-π interaction.

A change in pressure as seen from Table 6.4 achieved approximately 10% reduction in retention factor and reduced or increased the resolution by about 2 - 5%. In order to reduce the retention factors (40-70%) the modifier needs to be changed by about 15%. To achieve a similar change using pressure, a pressure of 400-550kg/cm^2 would be required to achieve 40-70% change in retention factor. High pressures, such as this were not feasible with the instrument available, as the UV high pressure cell could only be exposed to pressures up to 300 kg/cm^2.

It can however be deduced from the decrease in resolution with increasing modifier concentration that increasing the pressure is a more efficient way to decrease retention. To obtain optimised separations it is advisable to begin with the highest possible pressure and then adjust the retention times by varying the retention factor by increasing or decreasing the modifier concentration.

Table 6.4 Comparison of the influence of modifier and pressure on retention and resolution

Chromatog. Parameter	Modifier concentration	Modifier	Pressure
	Range		200-250kg/cm²
	[%]	Change in [%]	
k (P1a)[a]	10-25	-68.0	9.5
k (P2a)	8-20	-61.7	-9.5
k (C3a)	20-35	-42.2	-9.8
k (C4a)	20-35	-41.7	-11.3
k (C5a)	8-20	-60.4	-10.4
k (C7a)	20-40	-44.4	-13.5
k (C8a)	10-25	-76.8	-12.7
k (C9a)	10-30	-45.5	-11.6
k (C11a)	20-40	-43.7	-12.4
R_S (P1a- P1b)	10-25	-41.3	+0.6
R_S (P2a- P2b)	8-20	-49.5	+2.0
R_S (C3a- C3b)	20-35	-30.4	-1.1
R_S (C4a- C4b)	20-35	-35.8	+3.1
R_S (P5a- P5b)	8-20	-47.1	-4.5
R_S (C7a- C7b)	20-40	-24.0	-2.9
R_S (C8a- C8b)	10-25	-21.8	+2.0
R_S (C9a- C9b)	10-30	-26.9	-4.4
R_S (C11a- C11b)	20-40	-29.9	+0.6

6.3.3 Future Work

It would be worthwhile trying to elucidate the chiral recognition mechanism present on (S)-NEC-β-CD and on β-CD columns, in order to be able to assess possible applications of chiral SFC using cyclodextrin columns. If the results using SFC can be compared with either the NP, RP or polar organic mode, it would be possible to predict possible applications using cyclodextrin columns, or if no

correlation between SFC and the LC separations is established, SFC offers a unique separation mechanism. The use of analogues is recommended for a systematic study so as to elucidate the retention mechanisms in each of the techniques.

It would be interesting to investigate whether the substitution of the iso-PA with glacial acid or a combination of the two would have a major impact on the resolution for the clofibrate analogues or other chiral compounds separated in the polar organic mode on CD columns. Furthermore, this may achieve faster resolution of the compounds, since the resolution should not deteriorate to the same extent with increasing modifier.

6.2.4 Bn-ether Investigation

The analysis of the Bn-ether on the (S)-NEC-β-CD is summarised in Table 6.5 in the same way as the previous results. The level of MeCN achieved a 50% change in retention time by increasing the modifier content from 20 to 25%, however resolution was reduced concomitantly by 25%. Selectivity was also influenced by the concentration of modifier.

An even more pronounced effect on retention and selectivity was achieved by variation of the MeOH level in the MeCN modifier. Retention and selectivity changed by 70% and 3% respectively. Resolution also decreased, although to a lesser degree than with the variation of MeCN concentration, since the same loss of resolution was experienced, however a greater change in retention was achieved.

Temperature, together with the changing MeOH level was the most efficient parameter to change selectivity, which was expected according to the predictions given by Berger (1995). A minimum in retention was achieved with increasing temperature (55°C), thus showing the trend predicted by Berger (1995), which is the opposite to retention behaviour with increasing temperature on capillary columns. Table 6.4 demonstrates that the increase in temperature is beneficial for

the separations, as the separation is accelerated, while the resolution and selectivity is improved.

Table 6.4 Comparison of the influence of physical parameters on the separation of Bn-ether

Parameter	Range	k	Rs (Bn a-Bn b)	α (Bn a-Bn b)
			Change in [%]	
MeCN [%]	20-25	-49.3	25.4	1.0
MeOH [%]	0-15	-69.6	26.7[a] (-44.9)	-3.1
Temperature [°]	25-75	-5.6[b] (7.0)	7.3[c] (-6.3)	0.9[c] (1.9)
Pressure [kg/cm²]	150-250	-35.6	-9.5	-0.5
Flow rate [ml/min]	0.5-1.0	-14.9	-24.1	-0.4

[a] increase observed from 0-2.5% MeOH, significant decrease from 10-15% MeOH.
[b] decrease from 25-55°C, increase from 55-75°C.
[c] increase from 25-45°C, decrease from 45-75°C.

Pressure was again a good method to influence retention to a moderate degree without decreasing the resolution and selectivity to a great extent, therefore it is recommended to start separations with reasonably high pressures of 200-250kg/cm². Higher pressures are not recommended with this particular instrument, since the maximum pressure was 300kg/cm².

A change in flow rate changed resolution most significantly, compared to the changes achieved in retention and selectivity. It is therefore recommended to choose optimum flow rate conditions and accelerate a given separation by varying the modifier concentration, since higher retention time changes can be achieved without the same loss in resolution.

To sum up, it was unexpected that a mixture of two modifiers resulted in an improved resolution and a considerable time saving in achieving the separation. It has been known that MeCN is the more efficient modifier in achieving a desired

separation and MeOH can be used to adjust the retention. The same principle was used in the thesis of Matchett (1996) when investigating CD as stationary phase in the polar organic mode. This further suggests that in future there should be trials in which MeCN is applied in chiral separations to achieve better selectivity. However, solubility might be again a problem, so that the addition of MeOH may be necessary.

The separation of the diastereoisomers was superior on the (S)-NEC-β-CD than on the β-CD column and no resolution was observed on the diol or silica column. This demonstrated that the (S)-NEC-β-CD column is not only an advantage for specific chiral separations, but can also be used for the separation of diastereoisomers.

6.4 Supercritical Fluid Extraction

6.4.1 Fatty Acid Extraction

The extraction of fatty acids from cotton seed meal and soya meal was optimised using the expert design system, and an optimisation was achieved within 15 experiments. The subsequent evaluation pinpointed the conditions at which optimum extraction took place using the combination of dynamic and static extraction procedures. Staby and Mollerup (1993) reviewed the extraction of fatty acids from fish oil and the fatty acids could be easily extracted using pure CO_2. However, when pure CO_2 was used in this work, the recoveries were only about 2-50% in the given time limit as shown in Table 5.2. Dean and Lowdon (1993) extracted megestrol acetate from a tablet matrix using a Hewlett-Packard and a Jasco system. They observed a far lower recovery using almost identical conditions on the Jasco system, which was thought to be due the number of cell volumes swept. When modifier was used (10%), the recovery improved by 30%, however

this was still lower than the Hewlett-Packard instrument using pure CO_2. The reason for the reduced recovery for megestrol acetate and the fatty acids may have the same reason, namely precipitation of the extracted compounds in the needle valve of the backpressure regulator and insufficient flushing of the compounds into the trapping solvent. The Hewlett-Packard system used an electronically actuated variable restrictor and the extracted compounds were collected onto a chromatographic support material. The collection column may still cause a certain amount of backpressure, which is possibly enough to transport the extracted megestrol acetate to the support material, whereas in the Jasco system the depressurisation takes place within the needle valve. This hypothesis was further supported with the fractionation of the PCBs, which clearly showed that the PCBs precipitated within the backpressure needle valve. The collection efficiency of the fatty acids were not determined at the time, however it was difficult to determine if the poor recovery was due to poor solubility of the compounds in the pure fluid or if the extracted compounds precipitated in the needle valve and were not transferred into the collection solvent. This could however be investigated by the use of a third pump (as used in section 3.4.2), which is added to the system before the backpressure regulator to assist the transfer of the compounds to the collection solvent.

In addition to the problem of transfer of the fatty acids, good solubility was required initially to extract the fatty acids present at the matrix surface. Using 9mol% modifier, 92% of the fatty acids were extracted in the first 15 minutes, which means that high solubility was necessary early on in the extraction. However, the solubility of fatty acids should be sufficient as determined by Maheshwari et al. (1992), therefore the recovery limit must have been due to the precipitation of the fatty acids in the needle valve.

6.4.2 Nicotine Extraction

The hot-ball model applied by Bartle et al. (1992a) was useful to evaluate the influence of different physical parameters, although virtually the same conclusion could have been drawn from plotting recovery versus time. However, the model allows the estimation of the original amount in the sample, unless the compound is locked into the matrix such as in the example given by Clifford (1993) for the extraction on cylic trimer from a PET film. It is therefore recommended to compare the value obtained from experiments, in which various extraction techniques are used. As shown for the nicotine extraction there was an excellent correlation between the values obtained from liquid extraction and the SFE results. Hawthorne (1993) further recommended subsequent extraction techniques on one sample to ensure that quantitative extraction took place.

It is difficult to propose the optimum method to develop a SFE method, as the purpose of the extraction has to be known. If a fast extraction is desired in which the co-extraction of other compounds is not important, the extraction conditions can be optimised to reach maximum solubility. The flow rate however has to be low enough to enable quantitative collection. In contrast, if selective extraction is required, it is important to find the appropriate extraction conditions which allow the selective extraction of the compound.

To sum up, before new methods are developed on the Jasco SFE extraction system, an improvement of the present system in terms of transfer of the extracted compounds to the collection solvent is required. This can be achieved either by the addition of a third pump as described in section 3.4.2 or by the use of solid phase trapping, in which depressurisation takes place across the solid phase trapping column. The use of modifier however posses a problem in the solid phase trapping system, since it may rinse the extracted compounds from the trap.

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